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(AGRICULTURAL RESEARCH SECTION)

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## NOTICES

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THE NEW ZEALAND

# JOURNAL OF SCIENCE

AND

# TECHNOLOGY

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VOL. 30. SEC. A FEBRUARY, 1949 NUMBER 5

## STUDIES IN MONOZYGOTIC CATTLE TWINS:

## I. ORGANIZATION OF TWIN COLLECTION

By JOHN HANCOCK, Ruakura Animal Research Station, Department of Agriculture, Hamilton

(*Received for publication, 8 April, 1949*)

### *Summary*

(1) The organization of twin collection work at the Ruakura Animal Research Station is described.

(2) In five years, 1944-8 a total of 222 sets of monozygotic twins have been located. This annual number of 40-50 sets is adequate to justify large scale use of monozygotic twins for experimental studies.

(3) Methods of calculation of frequency of monozygotic twinning are described with reference to data of Johansson and Bonnier Frequency rates are shown to be of major importance in twin collection work.

(4) The relation of distance, sex, collection methods, and year to the ratio of monozygotic twins in the inspected sample of cattle twins is discussed, in view of the usefulness of such data to workers attempting twin collection.

(5) Three factors on the organization side are essential to successful twin collection: (i) A relatively concentrated cattle population; (ii) Full time responsibility of a specialized worker; (iii) Enthusiastic farmer co-operation.

*"A fundamental characteristic of living organisms is that they are alike in general plan, and different in detail. There are two corresponding schools of biologists, one of which emphasizes the average, and the other, the individual and particular. The rapid development and diffusions of statistical techniques in the biologic as well as in the physical sciences, tends to emphasize the average and the general: on the other hand, great discoveries and concepts of biology have resulted from scrutiny of the individual, of the detail. . . . . ."*

S. BRODY *

### INTRODUCTION

THE difficulties of controlled experimental work with cattle, spring mainly from two sources: (1) The inherited variability in most economically important characteristics, and (2) their great size. For practical reasons their size prevents the inclusion of a sufficient number of individuals to level out the uneveness of the experimental groups due to the great natural variability of the animals concerned. Thus sampling errors tend to be excessively high. The advent of modern statistical methods strongly emphasises this dilemma. In consequence,

* Bioenergetics and Growth (1945)

the general tendency of recent years has been for a research worker on cattle to concentrate on large scale field investigations rather than on controlled experimental work. However useful as indications to a problem the results of such survey work may be, they are often merely descriptive of a general situation and rarely gauge the effect of a specific factor, or a combination of such factors on a cattle population. Only the direct experimental approach can yield a precise analysis of particular causes and their effects.

Some of the success of plant research work can be attributed to the fortuitous situation that pure lines and clones have been available as experimental material. Such pure lines occur naturally in the form of self-fertilized plants. Some plants which normally are cross fertilized can be artificially self-pollinated. By this method, pure lines which do not occur spontaneously can be obtained rapidly. A comparable situation yielding homozygotic experimental material as clones is obtained by grafting, budding and by other methods of vegetative reproduction. Although pure lines of the higher animals can be produced, the difficulties are unfortunately greater than with plants. This is mainly due to three factors:

(1) Self-fertilization is not possible.

(2) Intervals between generations are long.

(3) Cost of keeping animals compared with plants is great.

That pure lines of mammals can be produced, has been shown by the success obtained with the Wistar Institute Rat, by King with mice and Wright with guinea pigs. Some progress has also been made with pigs at Cambridge and at U.S.A. experimental stations. However, in spite of the evidence that intensive inbreeding is not necessarily detrimental to the vigour of animals, it is not likely that 100 per cent. inbred strains of dairy cattle will ever be approached. This becomes clear when it is considered that it would take ten generations or at least forty years, to obtain a line of cattle with an average inbreeding of approximately 87 per cent. (1). Many such lines may have to be started to find the one capable of development. In any event, animals of only one such line would be of limited usefulness for work on economically important problems, since, through such a line, the response of various environmental factors can be measured on only one specific genotype. It appears to be an almost impossible task to develop several such lines, each one representing a certain level of inherited productive capacity.

In view of these difficulties it is the more fortunate that artificial production of pure lines of dairy cattle need never be undertaken as their equivalents occur naturally as monozygotic twins.

The occurrence of monozygotic twins in man has been known for a long time and their potential usefulness as material for studies of genetical problems has been widely recognized (2). Although five sets of presumably monozygotic cattle twins had previously been described in the literature, it was not until 1932 that Kronacher (3), in an exhaustive study of 35 sets of like-sexed twins, finally demonstrated that monzygotic twins do occur in this species. Kronacher (4), summarizing his work, described 11 sets of monozygotic twins. Haak (5), described another eight sets, Chapman *et al.* (6), described one set and Lush (7), and Deakin (8), one each. Bonnier (9) (10), began research on cattle twins in Sweden in 1937 and in the four years 1941-4 he acquired for the Institute of Animal Breeding at Wiad, 34 sets.

Impressed by the possibilities arising from Bonnier's pioneer work, and following a visit to Sweden in 1938, McMeekan (11), initiated a search for monozygotic twins in New Zealand in 1941. On behalf of the Ruakura Animal Research Station, Dry of Massey College assumed responsibility for the North Island, and McMeekan for the South Island. Ruakura was able to receive one set of twins and one set of triplets in 1942, and four sets in 1943, mainly as a result of Dry's efforts. They were acquired when a few days old. Altogether Dry located 12 older sets up to 1944. In the South Island, thirteen sets of like-sexed twin calves were examined, none proving monozygotic. One set of four-year-old Shorthorn cows was located. Work was abandoned in the South Island because of the few dairy cattle available and the wide dispersal of the cattle population necessitating excessive travel under war-time conditions of transport. In 1944 the Ruakura Animal Research Station decided to begin a drive to ascertain whether a sufficient number of monozygotic twins could be acquired to make long term research, using such animals as material, a worthwhile undertaking. Though results had been disappointing to date it was considered that special organization on the part of a full time worker might give more promising results. The author was made responsible for the project.

## METHOD OF COLLECTION

The advantages and difficulties of collection of twins under New Zealand conditions can be appreciated only if some of the peculiarities of the New Zealand dairy industry are considered. Because the dairy farmers of the North Island, where over 80 per cent. of the cows are located, rely almost exclusively on grass and grassland products as fodder for their cattle, the calving season is restricted to a relatively short period in the early Spring. The shortness of the calving season has two advantages from the point of view of collection of monozygotic twins:

(1) Twin calves collected in any one year are more or less of the same age.

(2) The work of collection does not necessarily interfere with other activities of a research worker as would be the case if calving were distributed evenly throughout the year.

It is easy to understand that as the calving season is the busiest time of the year for a dairy farmer in New Zealand he cannot undertake any tasks which are not strictly necessary to the orderly running of his farm. He cannot be expected to keep twin calves for inspection longer than the four days for which the law requires him to hold all calves before he sends them to the freezing works. It should be noted that most twin heifers and all twin bulls from commercial herds are disposed of as "bobby calves" or veal, when 4-7 days old. Early season twin heifers may be kept for replacement purposes and the farmer generally insists on having these inspected as soon as possible, so as to know how many more heifer calves he must retain should the inspected animals prove monozygotic and be sold for experimental purposes. As the majority of farmers are not prepared to make arrangements to send twin calves by rail, owing to the extra trouble involved, these must be collected on the farm.

In view of these difficulties which were increased by war-time transport shortages, it was decided to make an attempt to inspect twin calves upon their arrival at the freezing works. The Ruakura Animal Re-

search Station is situated near Hamilton in the midst of one of the most intensive dairying areas in the world. A circle, with Ruakura as centre, and with a radius of twenty miles, includes approximately 160,000 dairy cows in milk or approximately 10 per cent. of the whole dairy cow population of the Dominion. A circle of forty miles radius includes 320,000 cows (12) (see Fig. 1). Most of the surplus calves from this area are slaughtered at the Auckland Farmers' Co-operative Freezing Works at Horotiu, situated ten miles from Ruakura. The arrangement for collection and transport of the calves are made by farmers' co-operative organizations known as " Bobby Calf Pools ". At these works, 170,000 calves are slaughtered annually, and as dairy farmers in New Zealand keep approximately 20-25 per cent. of all calves for replacements, this represents a dairy cow population of at least 230,000 cows.

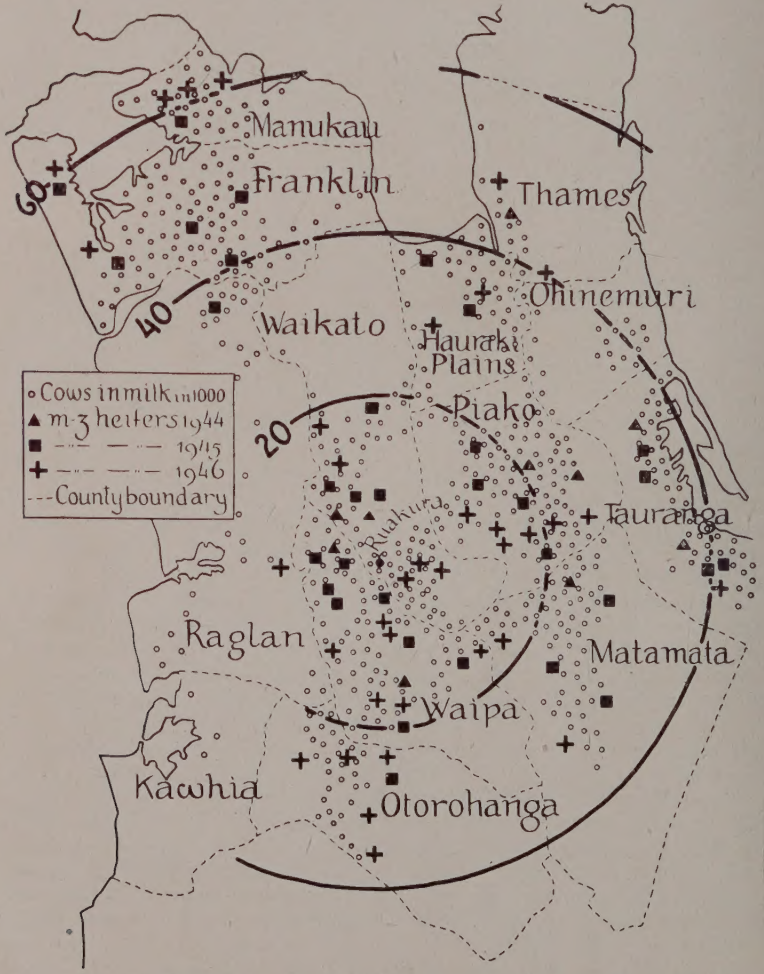


FIG. 1.—Distribution of the dairy cow population in the South Auckland and part of the North Auckland Land Districts, and the localities in which monozygotic twin heifers were found.

Through advertisements, and articles in the local press and agricultural journals, and per medium of the radio, farmers sending calves to Horotiu were asked to paint and label any like-sexed twins they thought were of monozygotic origin and to inform the drivers of the "Bobby Calf" lorries that the calves were twins which had to be sorted out on arrival at the Freezing Works for inspection by an officer from Ruakura. If the calves were to be transhipped by rail the lorry driver had to note on the railway consignment form that twins were included. This was necessary as it was not possible to arrange for a thorough search for eventual twins in every truck-load of calves that arrived at the Works. Farmers within a reasonable distance from Ruakura were asked to communicate directly with the Animal Research Station, and a promise of prompt inspection of the calves was made.

In the 1945 season the same method of collection was employed. In addition to the newspaper appeal, a leaflet containing advice on preliminary recognition of monozygotic twins was sent to practically all farmers supplying the Horotiu Freezing Works.

In view of the relatively few twins inspected at the Freezing Works in the previous year, (See Tables IVA and IVB) it was decided in 1946 to inspect all twins on the farm. A leaflet was again distributed. This method has proved very successful and has since been followed. It will be of interest to workers involved in twin collection to note the comparative failure of the approach through the Freezing Works. Initially, this appeared an ideal system but even in the first year when it was the only method advertised it accounted for only 50 per cent. of the twins examined. In the second year, the proportion fell to 20 per cent. During these two years it became increasingly clear that the factor of personal contact between research worker and farmer was of major importance. Farmers were more prepared to co-operate if and when they found the research worker was sufficiently interested to visit the farm and inspect the animals on the spot. Merely to despatch the animals to the works with labels attached was too impersonal, and appealed only to those interested in the greater financial return, should the calves prove monozygotic. On the other hand, personal inspection resulted in a situation where the farmer believed he was making a real and personal contribution to research.

## NUMBERS COLLECTED

The following is a short summary showing the number of monozygotic twins of both sexes collected in these three seasons.

TABLE I. MONOZYGOTIC TWINS COLLECTED

| | Heifers. (sets) | Bulls. (sets) | Total. (sets) |
|---|---|---|---|
| 1944 ... ... ... | 15 | 9 | 24 |
| 1945 ... ... ... | 38 | 8 | 46 |
| 1946 ... ... ... | 37 | 5 | 42 |
| 1944/46 ... ... ... | 90 | 22 | 112 |

Prior to transferring five sets of heifer calves to the Dairy Research Institute, Palmerston North, in the Spring of 1946, the population of monozygotic twins at Ruakura reached 90 sets. Thus sufficient numbers of monozygotic twins can be located in New Zealand to make

possible large scale projects, using these animals as experimental material. Collection work in 1947 and 1948 resulted in a further 99 sets of heifers, and eleven sets of bulls, making a total of two hundred and twenty-two sets located in the five years.

The number of monozygotic twins collected in the last three seasons was considered sufficient for the immediate needs. Based on an estimated requirement of 30 sets in milk, and 20 sets under special experimental conditions, the yearly replacements are calculated to be of the order of 25 to 30 sets, and it is considered that this number can be acquired easily without enlarging the present organization. If, however, a need for a larger number of monozygotic twins arises in the future, or if other research institutions decide to use such animals for experimental work, it is useful to know that there are three other areas in New Zealand apart from the Waikato, with a sufficiently large and concentrated cow population to be suitable sources of twin calves. These areas are: (1) North Auckland, with 354,000 cows; (2) Taranaki, with 215,000 cows; and (3) the Manawatu-Wellington area, with approximately 212,000 cows (12).

Finally, it may be pointed out that an intensification of the search for twins in the Waikato may result in a substantial increase in the number of monozygotic twins found annually. Fig. 1 has been prepared to show the density of the cow population in the counties within 60 miles of Ruakura. The location of the source of each set found during the search in the years 1944-6 has also been marked. If all counties had contributed the same number of twins relative to cow populations as the Waipa County a further 56 sets of heifer twins would have been acquired.

## FREQUENCY OF MONOZYGOTIC TWINS

The frequency of monozygotic twins in a cattle population has a direct bearing upon the potential number available and upon the ease of collection. The number available is measurable by the ratio of monozygotic twins to total calvings, so that an assessment of this frequency is of primary importance. The ease of collection is related very closely in practice to the ratio of like-sexed twins to monozygotic, since the ratio determines the number of like-sexed twins that have to be examined for every monozygotic set located. Since the overall twinning rate in a cattle population directly affects the number of like-sexed twins born, it is rather important to determine whether there is a constant relationship between the overall twinning rate and the monozygotic twinning frequency. At first sight, a high overall twinning rate should make monozygotic twin collection easier, both in respect to numbers and differentiation. This will not be the case unless this increase is accompanied by more than a proportionate increase in the monozygotic twinning frequency, since without this, the number of twins that have to be examined for sets of monozygotic twins located is increased. Ideally, twin collection will be easy both in respect to number available, and ease of collection, if the monozygotic twinning frequency is high and the overall twinning rate is low.

Though the point has not been discussed by these authors, it is clear from the data of Johansson (13), and Bonnier (10) (14), that the overall twinning rate in cattle gives no indication of the rate of monozygotic twins in the same population. Working with three breeds in Sweden, both authors have collected data on the distribution of like-and unlike-sexed twins, while Johansson has reported the overall twinning frequency for these same breeds. By using the method

employed by Strandkov *et al.* (15), of subtracting the like-sexed from the unlike-sexed twins, calculation of the number of monozygotic twins occurring in these samples has been attempted. This fifty-fifty method has given identical results in a human population to that which takes the sex ratio of twins into consideration (14). The results of applying the method to the Scandinavian material are shown in Table II.

TABLE II. CALCULATED FREQUENCIES OF MONOZYGOTIC TWINS IN SWEDISH CATTLE BREEDS

| Basis. | Swedish Fresian. | | Swedish Red and White. | | Swedish Polled Landrace. | |
|---|---|---|---|---|---|---|
| | Johansson. | Bonnier. | Johansson. | Bonnier. | Johansson. | Bonnier. |
| Whole population | 0.054 | 0.084 | 0.128 | 0.10 | 0.243 | 0.290 |
| All twins | 1.62 | 2.50 | 6.94 | 5.34 | 13.43 | 16.00 |
| Like-sexed twins | 3.19 | 4.87 | 12.98 | 10.14 | 23.68 | 27.59 |
| Overall-twinning per cent. | 3.35 | — | 1.85 | — | 1.81 | — |

It will be noted that the Swedish Fresian breed, showing the highest overall twinning rate of 3.35 per cent. shows the lowest overall monozygotic twinning frequency of 0.54 to 0.84, while the Swedish Red and White and Swedish Polled Landrace with overall twinning rates of 1.85 and 1.81, show markedly different overall monozygotic frequencies. Both sets of data show comparable trends so that despite Bonnier's conclusion that the inter-bred differences in his own data were not statistically significant, it would appear that the ratio of the overall twinning rate to the monozygotic twinning frequency is by no means a constant. In arriving at his conclusion, Bonnier worked only on the ratio of monozygotic twins to all like-sexed twins and did not take into account Johansson's material. The point is of fundamental importance since it is related so closely to the difficulties of twin collection work. If Bonnier's conclusions are sound, the success of twin collection in New Zealand could be repeated in any cattle population. If, however, the view expressed above is valid, viz. that the rate of monozygotic twinning is not constant relative to the overall twinning rate, then the ease of twin collection will be quite variable, depending on the ratio of the two twinning rates in the cattle population under consideration. In this connection it is important to point out that the overall twinning frequency in New Zealand is very low, (0.60 to 1.0 per cent.) but presumably, the monozygotic twinning frequency is reasonably high. Data printed later suggests a rate of 0.1 per cent. for monozygotic heifer twins. The only New Zealand data available for twinning rates in cattle is that of Ward (16) (17). His analysis of a sample of approximately 27,000 calvings in 1945-6 gave the following results.

TABLE III. INCIDENCE AND SEX DISTRIBUTION OF TWINS

| | Two Males. | Male and Female. | Two Females. | Total. |
|---|---|---|---|---|
| Twin Births | 63 | 117 | 82 | 267 |
| Total Calvings | | | | 26,976 |
| Twinning Rate per cent. | | | | 1.0 |

Calculation of the frequency of monozygotic twins from these data is rather dangerous in view of the markedly abnormal distribution of the three sex combinations, indicating an inexplicable deficiency of males. Ward (16), made such an estimate and reported a probable monozygotic twinning rate of 0.1 per cent. of all births. Unfortunately, application of the same method to Ward's data for 1946-7 (17), on a comparable sample gives a surplus of like-sexed twins of only one set in 25,400 calvings, a large proportion of which were located in the Ruakura territory. In this latter data a similar marked deficiency of males existed, while in the same season Ruakura collected 42 sets of monozygotic twins. It is therefore obvious that no reliable estimate of the monozygotic twinning frequency for New Zealand cattle can be obtained from Ward's material. Thus little weight can be given to Korkman's (18) use of these data to study twinning frequencies or to his conclusion that no breed differences exist in the monozygotic ratio. Strandkov's (15) analysis of monozygotic twinning frequencies in white and negro populations in the U.S.A. shows statistically significant racial differences. It is perhaps important to point out that differences of the type recorded here for cattle may have a regional, rather than a racial basis, the three breeds occupying markedly different regional areas in Sweden.

## THE SIRE AS A POSSIBLE CAUSE OF MONOZYGOTIC TWINNING IN CATTLE

Although it is not intended to discuss generally the causes of monozygotic twinning in cattle, it seems worthwhile in this connection to put on record evidence that the sire, in some cases, may be involved. The evidence is based on four cases of bulls, each having sired two sets of monozygotic twins. In view of the low frequency of monozygotic twin births, these occurrences can hardly be ascribed to chance. Specific environmental factors obtaining on the farms concerned can also be ruled out since, considering the seasonal nature of calving in New Zealand, the twins were born at widely spaced time intervals.

The birth dates of the calves are set out below :—

| Bull | Set No. | Birth Dates |
|---|---|---|
| 1 | I | 17/7/45 |
| | II | 25/9/45 |
| 2 | III | 9/7/47 |
| | IV | 13/7/47 |
| 3 | V | 12/8/47 |
| | VI | 15/9/47 |
| 4 | VII | 30/9/47 |
| | VIII | 18/9/48 |

It can be seen that the two sets of twins sired by Bull 4 were born almost a whole year apart.

The conclusion seems inescapable that the sperms of a bull may be so constituted as to cause the fertilized ovum to split.

## ANALYSIS OF THE RUAKURA SAMPLE

Data have been collected on the frequency of monozygotic twins amongst the like-sexed twins which have been made available for inspection by farmers in New Zealand. It must be emphasized at the outset that the like-sexed twins which have been inspected did not represent a

random sample of all like-sexed twins born. Because farmers were asked to report only those like-sexed twins which were of similar appearance, especially as to colour and size, it is believed that the sample inspected contained a greater proportion of monozygotic twins than a truly random sample. Thus, the data are not suitable for calculation of the frequency of monozygotic twins in the twin population as a whole, but when analysed as to variations due to the sex of twins, year of collection, method of collection and location, they yield some interesting information about the ratios of like-sexed twins to monozygotic twins in an inspected sample. Such information may prove very useful to other workers in this field and is therefore presented here in some detail.

Tables IVA and IVB show the ratios of twins inspected to monozygotic twins for the three seasons concerned. The data for 1944 are not complete in that no information is available as to the distance from Ruakura travelled by the twins which were inspected at Horotiu. These data are also incomplete insofar as they cover only the first 96 sets of twins inspected that year.

### DISTANCE FROM RUAKURA

The distance to Ruakura by road or rail from the farms where the twins were located strongly influenced the ratio of all twins to monozygotic twins in that the further from Ruakura sets of twins were located, the greater were the chances that they were monozygotic. The situation is graphically presented in Fig. 2.

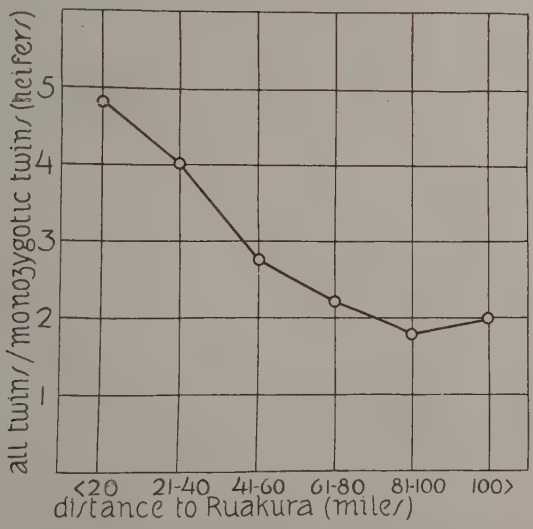


FIG. 2.—Relationship between distance of Ruakura from farms where twins were located, and the ratio of twins inspected to monozygotic twins.

The most likely explanation for this phenomenon is that farmers living further away from Ruakura tended to report a more selected sample of twins than farmers nearer to Ruakura. It is obvious that

A. HEIFERS

TABLE IV. TWIN COLLECTION DATA

| Distance from Ruakura. Miles. | Inspected on Farms. 1944 a | b | 1945 a | b | 1946 a | b | Total. a | b | Arrived by Rail. 1944 a | b | 1945 a | b | Total. a | b | Inspected at Horotiu. 1944 a | b | 1945 a | b | Total. a | b | Total 1944 a | b | Total 1945 a | b | Total 1946 a | b | Grand Total. a | b |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 20 or under | 20 | 3 | 38 | 6 | 37 | 11 | 95 | 20 | 0 | 0 | 0 | 0 | 0 | 0 | x | x | 6 | 1 | x | x | x | x | 44 | 7 | 37 | 11 | 101 | 21 |
| 21-40 | 2 | 2 | 29 | 8 | 40 | 10 | 71 | 20 | 1 | 0 | 2 | 0 | 3 | 0 | x | x | 6 | 0 | x | x | x | x | 37 | 8 | 40 | 10 | 80 | 20 |
| 41-60 | 0 | 0 | 3 | 0 | 20 | 7 | 23 | 7 | 3 | 1 | 6 | 4 | 9 | 5 | x | x | 1 | 0 | x | x | x | x | 10 | 4 | 20 | 7 | 33 | 12 |
| 61-80 | 0 | 0 | 11 | 5 | 12 | 7 | 23 | 12 | 2 | 1 | 7 | 2 | 9 | 3 | x | x | 5 | 2 | x | x | x | x | 23 | 9 | 12 | 7 | 37 | 17 |
| 81-100 | 0 | 0 | 0 | 0 | 3 | 1 | 3 | 1 | 0 | 0 | 4 | 3 | 4 | 3 | x | x | 0 | 0 | x | x | x | x | 4 | 3 | 3 | 1 | 7 | 4 |
| 101 or more | 0 | 0 | 7 | 4 | 1 | 0 | 8 | 4 | 4 | 2 | 6 | 3 | 10 | 5 | x | x | 0 | 0 | x | x | x | x | 13 | 7 | 1 | 0 | 18 | 9 |
| Total | 22 | 5 | 88 | 23 | 113 | 36 | 223 | 64 | 10 | 4 | 25 | 12 | 35 | 16 | 22 | 2 | 18 | 3 | 40 | 5 | 54 | 11 | 131 | 38 | 113 | 36 | 298 | 85 |
| Ratio per cent. | 4.4 | | 3.8 | | 3.1 | | 3.5 | | 2.5 | | 2.1 | | 2.2 | | 11.0 | | 6.0 | | 8.0 | | 4.9 | | 3.4 | | 3.1 | | 3.5 | |

B. BULLS

| Distance from Ruakura. Miles. | Inspected on Farms. 1944 a | b | 1945 a | b | 1946 a | b | Total. a | b | Arrived by Rail. 1944 a | b | 1945 a | b | Total. a | b | Inspected at Horotiu. 1944 a | b | 1945 a | b | Total. a | b | Total 1944 a | b | Total 1945 a | b | Total 1946 a | b | Grand Total. a | b |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 20 or under | 14 | 2 | 14 | 3 | 13 | 1 | 41 | 6 | 0 | 0 | 0 | 0 | 0 | 0 | x | x | 4 | 0 | x | x | x | x | 18 | 3 | 13 | 1 | 45 | 6 |
| 21-40 | 0 | 0 | 10 | 0 | 19 | 2 | 29 | 2 | 1 | 0 | 2 | 0 | 3 | 0 | x | x | 6 | 1 | x | x | x | x | 18 | 1 | 19 | 2 | 38 | 3 |
| 41-60 | 0 | 0 | 1 | 0 | 2 | 1 | 3 | 1 | 2 | 0 | 1 | 0 | 3 | 0 | x | x | 3 | 2 | x | x | x | x | 5 | 2 | 2 | 1 | 9 | 3 |
| 61-80 | 0 | 0 | 2 | 1 | 0 | 0 | 2 | 1 | 0 | 0 | 2 | 1 | 2 | 1 | x | x | 0 | 0 | x | x | x | x | 4 | 2 | 0 | 0 | 4 | 2 |
| 81-100 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | x | x | 2 | 0 | x | x | x | x | 2 | 0 | 1 | 0 | 3 | 0 |
| 101 or more | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | x | x | 0 | 0 | x | x | x | x | 0 | 0 | 1 | 1 | 2 | 1 |
| Total | 14 | 2 | 27 | 4 | 36 | 5 | 77 | 11 | 4 | 0 | 5 | 1 | 9 | 1 | 24 | 4 | 15 | 3 | 39 | 7 | 42 | 6 | 47 | 8 | 36 | 5 | 125 | 19 |
| Ratio per cent. | 7.0 | | 6.8 | | 7.2 | | 7.0 | | | | | | 9.0 | | 6.0 | | 5.0 | | 5.6 | | 7.0 | | 5.9 | | 7.2 | | 6.6 | |

x Indicates data not available. a Shows number of sets inspected. b Shows number of monozygotic sets inspected.

the increasing difficulties either to the farmer himself or to the inspecting officer, made more distant farmers examine the twins more carefully.

The sample of heifer twins located within 20 miles of Ruakura, which may be considered the least selected one, provides a basis for an estimate of the maximum number of monozygotic twins born in the Waikato dairy cow population. Assuming that one per cent. of all calvings result in twins and that approximately 25 per cent. of all twins are heifer-heifer twins, it can be calculated that with one set of monozygotic twins in every 4.8 sets inspected, there are no more than one set of monozygotic heifers born in approximately 2,000 calvings.

## SEX

On analysing the data it soon became evident that bull calves had to be treated as a separate group, as they showed much lower ratio (1 : 6.6) than the heifer calves (1 : 3.5). There may be a number of possible reasons for this phenomenon of which the more likely ones are discussed below.

In view of the fact that, as shown above, the further from Ruakura sets of twins were located the greater were the chances that they were monozygotic, it was thought that if the bull twins were found to be located on farms on an average nearer to Ruakura than were the heifer twins, the differences in the respective ratios of twins inspected to monozygotic twins may not have been a real one. However, as 60.7 per cent. of the bulls compared with 66.4 per cent. of the heifers were inspected within 40 miles of Ruakura, this cannot have been a major factor causing the discrepancy in the ratios. That there was a real difference in the ratios for bull and heifer twins is also suggested by the fact that, of the heifer twins located on farms within 20 miles of Ruakura, which previously has been shown to be the least selected sample, one in every 4.8 was monozygotic compared with one in every 7.5 for the bulls.

Farmers may have tended to report bull twins on an average at a lower age than heifer twins, thus not having had the same opportunity to keep them under surveillence and, therefore, reported to Ruakura relatively more dizygotic bull twins than heifer twins. At first sight there seemed to be some grounds for this theory in that the average age (4.0 days) at which the bulls had been reported actually was slightly lower than that of the heifer twins (average 5.2 days). The distribution of the age classes in the two twin populations comprising 155 sets of heifers and 36 sets of bulls inspected in 1946 is shown in Fig. 3.* However, it has also been possible to calculate the proportion of monozygotic twins in the various age classes of heifers and as Fig. 3 shows, there is no apparent correlation between the age at which they were reported to Ruakura and the proportion of monozygotic sets. This does not however, exclude the possibility that some other factor of

* A set of pedigree bull twins which had been reported at age of three and a half months is not included in the above data as it was far older than any other set, and its inclusion would have given a false impression of the average age of the bull twins.

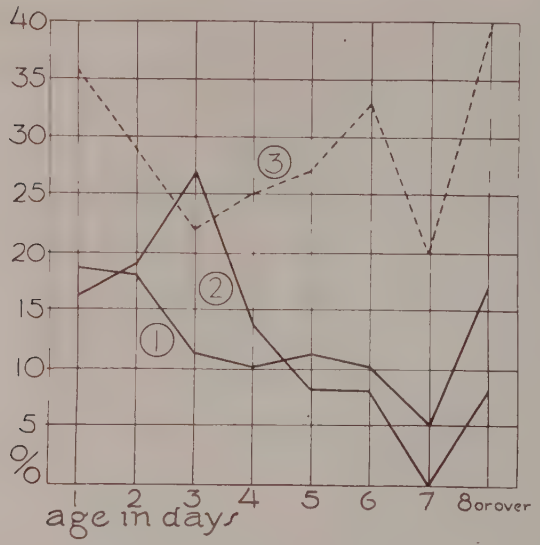


FIG. 3.—(1) Frequency distribution of the ages at which twin heifers were inspected.

(2) Frequency distribution of the ages at which twin bulls were inspected.

(3) Percentage of monozygotic twin heifers calculated on all twin heifers inspected at various ages.

a psychological nature so influenced the farmers that they tended to report to Ruakura a more unselected sample of bull twins than of heifer twins.

Greater pre-natal or early post-natal death rates of monozygotic bull twins are, of course, factors which could explain the discrepancy. The only evidence that would tend to support this theory is the fact that the secondary sex ratio in cattle is lower in twins than in singles (Johansson [13] and Ward [16] [17]), a fact however, which may be due to various other reasons than that there are relatively fewer monozygotic bulls in the twin population. It is believed that only a direct inspection of a large sized unselected sample of like-sexed twins will clarify this question.

Because of the relatively small number of bull twins which have been inspected, the discussion will hereafter be limited to the heifers.

## METHOD OF COLLECTION

As previously mentioned, three different methods of collection were employed in 1944-5.

(1) Selection of twins on arrival at the freezing works;

(2) Selection of twins on the farms;

(3) Direct consignment of twins to the research station by the farmer.

The relative number of monozygotic twins was smallest in the first group, and greatest in the last, the ratios of all twins inspected

to monozygotic twins being 8.0, 3.9 and 2.2 respectively. This situation could probably best be interpreted as due to the reluctance on the part of the farmers to send *via* the freezing works the twins they were convinced were monozygotic and instead, asked for inspection on the farms. It is not possible to decide whether the high proportion of monozygotic heifer twins in the sample of twins that arrived by rail was due to the method of collection, or the fact that most of these twins came from farms situated forty or more miles from Ruakura.

## YEAR

It is important to have some idea as to whether availability of twins varies from year to year. There are two reasons why the 1944 sample cannot be compared with those of 1945 and 1946. Only the first 96 sets of twins are included in 1944. This may have had some bearing on the number of monozygotic twins found, as it is possible that later in the season the relative number of monozygotic sets may have increased or decreased. In addition, the twins were located on farms which were on an average nearer Ruakura than was the case in 1945 and 1946.

In the two comparable years there was slightly more monozygotic twins in 1946 than in 1945. One in every 3.1 sets of like-sexed heifer twins inspected in 1946 was considered monozygotic compared with one in every 3.5 in 1945. The difference is unlikely to be significant.

## ESSENTIALS OF ORGANIZATION

The comparative failure of the first four years of twin collection work as contrasted with its marked success in the last four years has emphasized the importance of certain aspects of organization. From the experience gained it is fairly evident that at least three factors on the organization side are important if twins are to be obtained in adequate numbers to permit their effective use as material for cattle experiments.

(1) A relatively concentrated cattle population.

(2) Full time responsibility (seasonal) of a specialized worker.

(3) Enthusiastic farmer co-operation.

In the first four years none of these essentials was fulfilled. The cattle populations worked with were relatively widely dispersed. The workers concerned could direct but a small amount of time to the task, and in practice were available merely for recognition purposes. Farmer interest on a wide scale could not be obtained, partly because the first two essentials could not be met, and partly because the pressure of the early war years made it difficult to interest farmers in a project, the potential worth of which was difficult to grasp and which appeared to be of dubious value in the face of possible invasion of New Zealand by enemy forces.

During the past five years, concentration on a large compact cow population by one worker with full responsibility for all phases of the work during a period when farmers were emerging from war time pressure so that they could be approached successfully on farming matters, provided a much more satisfactory background for collection. These points seem worth stressing in this general account since the poor results

in early years were almost responsible for abandonment of the project in New Zealand. It was only a firm belief in the very great potential value of monozygotic twins on the part of the controlling officers of the Animal Research Division, together with a realization of the practical difficulties involved, that resulted in the author being given the opportunity of full time organization of twin collection. These points should not be overlooked by institutions seeking this type of experimental material.

No reference has been made to the method of recognition of identical twins—obviously a factor of fundamental importance. This aspect is so wide in its scope and involves data so extensive that it will be published as Part II of this series of studies of monozygotic cattle twins.

## ACKNOWLEDGMENTS

The successful outcome of work of the character described in this paper depends naturally on the assistance and co-operation of a great many persons. Special thanks are due to Dr C. P. McMeekan, Superintendent of the Ruakura Animal Research Station, without whose enthusiastic and unfailing support this work could not have been carried out. It is a pleasant duty to acknowledge the willing co-operation of the officers of the various Calf pools and Horotiu Freezing Works. Thanks are also due to Mr M. N. Wallace who assisted the author in the later stages of this work. Finally, thanks are due to all farmers who made their calves available for inspection.

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# THE EFFECT OF ROOTSTOCKS AND INTERMEDIATE SCION VARIETIES ON THE COOL-STORAGE DISORDER, CORE-FLUSH, IN GRANNY SMITH APPLES

By C. A. S. Padfield,
Department of Scientific and Industrial Research, Wellington.

## *Summary*

Core-flush, a form of internal tissue collapse, is a major disorder of Granny Smith apples in cool store. Different root-stocks and intermediate scion varieties showed no consistent effect on the incidence of this disorder. There were wide seasonal variations in the incidence of core-flush, in successive crops from trees under test.

Granny Smith apples are particularly subject to core-flush in cool storage, this condition occurring earlier in the storage life of this fruit than any other disorder. In addition to normal Granny Smith plantings, many varieties have been reworked with this apple. Some of these varieties are themselves particularly prone to core-flush in cool storage, and it has been suggested that the inclusion of such intermediate scions might influence the amount of this disorder occurring in Granny Smith.

## Description

Core-flush is a cool storage disorder which affects the flesh between seed cavities, causing a red-brown discoloration. At first this is slight, but in extreme cases affected tissue becomes discoloured and desiccated while cracking within the core zone sometimes occurs. This is accentuated by holding fruit below the recommended temperature and is most common in immature fruit. Smock (1946) has shown that considerable reduction has been obtained when susceptible apples were withheld from cool storage for periods up to 20 days. From data as yet unpublished it has been found that a reduction has been obtained when Granny Smith apples were withheld from cool storage for periods of five to six weeks, but this is not a practical means of control. The varieties, Dunn's Seedling, Granny Smith, and Statesman were particularly susceptible, while it is found to a lesser extent in Cox's Orange, Delicious, Jonathan, and Sturmer Pippin.

## Experimental, 1944 and 1945

In 1944 and 1945 fruit from many combinations of rootstock—Granny Smith scion, and rootstock—intermediate scion variety—Granny Smith scion were collected and cool-stored. To obtain results under normal harvesting conditions, picking was left to individual growers to fit in with their own harvesting programme. With a few exceptions, all samples were picked within a few days of one another. Samples consisted of two bushel cases in 1944 and four bushel cases in 1945; all were stored at 34°F. and were removed from storage at approximately the same time. Every apple was cut in half and the degree of core-flush assessed. For record purposes four grades were used—"trace," "slight," "moderate," and "severe." The last two categories were classed as commercially significant. All samples were examined in 1944 between 24th and 28th of November, and in 1945 between 12th and 15th of November.

TABLE I. HASTINGS DISTRICT. PERCENTAGES OF TOTAL AND COMMERCIALLY SIGNIFICANT COREFLUSH ON REMOVAL FROM STORAGE.

| | | 1944 | | | | | | 1945 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Orchard. | Root Stock and Intermediate Scion Combination. | Total Apples per Sample. | Picking Date. | Storage Date. | Period of Storage (days). | Per cent. Total Coreflush. | Per cent. Commercially significant coreflush. | Orchard. | Total Apples per Sample. | Picking Date. | Storage Date. | Period of Storage (days). | Per cent. Total Coreflush. | Per cent. Commercially significant coreflush. |
| A | Northern Spy-Cox's Orange Pippin | 263 | 24/4 | 27/4 | 213 | 90 | 57 | A | 499 | 22/4 | 30/4 | 197 | 70 | 44 |
| B | Northern Spy-Dunn's Seedling | 250 | 20/4 | 27/4 | 213 | 89 | 25 | B | 497 | 23/4 | 30/4 | 197 | 84 | 48 |
| C | Northern Spy-Jonathan | 251 | 22/4 | 27/4 | 213 | 97 | 52 | C | 500 | 17/4 | 20/4 | 208 | 88 | 62 |
| D | Northern Spy-Delicious | 250 | 20/4 | 27/4 | 213 | 94 | 60 | I | 497 | 16/4 | 23/4 | 210 | 84 | 58 |
| E | Northern Spy-Statesman | 250 | 20/4 | 27/4 | 214 | 94 | 74 | — | — | — | — | — | — | — |
| F | Northern Spy-Sturmer Pippin | 250 | 21/4 | 27/4 | 213 | 92 | 57 | F | 500 | 25/4 | 30/4 | 195 | 79 | 58 |
| G | Northern Spy-Dougherty | 250 | 22/4 | 27/4 | 213 | 88 | 58 | J | 496 | 30/4 | 2/5 | 195 | 77 | 32 |
| H | Northern Spy-Reinette du Canada | 250 | 19/4 | 27/4 | 209 | 80 | 21 | H | 472 | 23/4 | 26/4 | 201 | 88 | 52 |
| | Northern Spy | — | — | — | — | — | — | K | 500 | 30/4 | 1/5 | 194 | 66 | 20 |

TABLE II. NELSON DISTRICT. PERCENTAGES OF TOTAL AND COMMERCIALLY SIGNIFICANT COREFLUSH ON REMOVAL FROM STORAGE.

| | | 1944 | | | | | | 1945 | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Orchard. | Root Stock and Intermediate Scion Combination | Total Apples per Sample. | Picking Date. | Storage Date. | Period of Storage (days). | Per cent. Total Coreflush. | Per cent. Commercially significant coreflush. | Orchard. | Total Apples per Sample. | Picking Date. | Storage Date. | Period of Storage (days). | Per cent. Total Coreflush. | Per cent. Commercially significant coreflush. |
| 1 | Northern Spy | 250 | 11/4 | 15/4 | 226 | 98 | 83 | 1 | 495 | 15/4 | 18/4 | 210 | 100 | 90 |
| | Large's Seedling | — | — | — | — | — | — | | 452 | 15/4 | 18/4 | 212 | 82 | 60 |
| 2 | Large's Seedling | 250 | 10/4 | 15/4 | 226 | 91 | 53 | 2 | 474 | 15/4 | 18/4 | 210 | 95 | 65 |
| 3 | East Malling XII | 238 | 24/4 | 27/4 | 213 | 87 | 25 | — | — | — | — | — | — | — |
| | East Malling XVI | 188 | 24/4 | 27/4 | 209 | 86 | 22 | — | — | — | — | — | — | — |
| 4 | Northern Spy-Cox's Orange Pippin | 250 | 17/4 | 21/4 | 220 | 98 | 70 | 4 | 471 | 13/4 | 20/4 | 208 | 95 | 74 |
| 5 | Northern Spy-Dunn's Seedling | 250 | 12/4 | 27/4 | 213 | 98 | 69 | 5 | 495 | 8/4 | 14/4 | 212 | 99 | 94 |
| 6 | Ivory's Double-Dunn's Vigour Seedling | — | — | — | — | — | — | 6 | 494 | 13/4 | 16/4 | 212 | 97 | 66 |
| 7 | Northern Spy-Jonathan | 250 | 20/4 | 27/4 | 213 | 97 | 66 | — | — | — | — | — | — | — |
| 8 | Northern Spy-Delicious | 250 | 11/4 | 15/4 | 226 | 96 | 63 | 8 | 499 | 11/4 | 16/4 | 215 | 99 | 93 |
| 9 | Northern Spy-Delicious | 250 | 17/4 | 21/4 | 220 | 98 | 78 | — | — | — | — | — | — | — |
| | Northern Spy-Sturmer Pippin | 250 | 17/4 | 21/4 | 219 | 96 | 62 | — | — | — | — | — | — | — |
| 10 | Northern Spy-King David | 250 | 17/4 | 21/4 | 220 | 90 | 33 | 10 | 496 | 21/4 | 28/4 | 197 | 90 | 55 |
| 11 | Northern Spy-King David-Washington | 250 | 24/4 | 27/4 | 209 | 82 | 45 | 11 | 500 | 7/5 | 15/5 | 181 | 78 | 36 |
| 12 | Northern Spy-London Pippin | 250 | 11/4 | 15/4 | 226 | 85 | 41 | 12 | 499 | 10/4 | 16/4 | 212 | 98 | 83 |
| 13 | Northern Spy-Statesman + Borax | 226 | 20/4 | 27/4 | 209 | 93 | 50 | — | — | — | | — | — | |
| | Northern Spy-Statesman No Borax | 226 | 20/4 | 27/4 | 213 | 85 | 26 | 12 | 502 | 10/4 | 16/4 | 214 | 91 | 66 |
| 14 | Northern Spy-Dougherty | 250 | 10/4 | 15/4 | 226 | 78 | 30 | 14 | 495 | 30/4 | 2/5 | 196 | 96 | 75 |
| 15 | Northern Spy-Reinette du Canada | 250 | 2/5 | 5/5 | 202 | 70 | 20 | 15 | 499 | 5/5 | 8/5 | 192 | 92 | 52 |

### RESULTS, 1944 AND 1945

Percentages of core-flush in the Hastings fruit are given in Table I, those for the Nelson samples in Table II.

Incidence of core-flush was high in all samples in both years, though the amount varied from year to year in fruit from the same trees. For any particular combination of rootstock and intermediate variety, variations between seasons and districts were considerable. Taking these variations into consideration, results indicate that the intermediate variety has little, if any, influence on the development of core-flush. Although dates of picking were all within the normal harvesting period for Granny Smith, it is possible that variance in maturity between orchards and differing time lag between picking and storing would account for some differences.

In one season where it was possible to compare fruit from trees which had received a dressing of borax of ½ lb to a tree as against similar fruit when borax was not applied, there was a tendency for core-flush to increase with borax application. A similar result was noted by Phillips and Johnston (1943).

### EXPERIMENTAL, 1946

Samples used in the previous two years showed wide and unexplained variations in the incidence of core-flush. It was considered possible that orchard management might play some part, as fruit had been collected from many different orchards. To test this point an attempt was made to select trees on the same rootstock in orchards under widely different types of management. Only two suitable blocks of trees could be found, these being Granny Smith on Northern Spy stocks. Orchard 1 was under good management, and the trees selected were superior to those of comparable age in Orchard 16 under poor management. Fruit from the only block of Granny Smith on Malling stocks was also included, as these stocks will be used for many future plantings in New Zealand. The trees on Malling stocks were in good condition and comparable with those on Northern Spy in Orchard 1. Four case samples were stored at 34°F. Picking and storage dates are shown in Table III. All fruit was examined between 24th and 31st of October.

### RESULTS, 1946

Figures obtained from the 1946 experiment are given in Table III.

Picking and storage dates differed slightly for the two samples from trees on Northern Spy. Sample 16 was picked two days earlier and received four days extra delay than Sample 1. It is unlikely that the difference shown in the Table could be attributed to earlier picking, but longer delay before storing could account for some of it.

Results from trees on Malling stocks were similar to those from trees on Spy. When considered with the figures for 1944 it appears that the introduction of Malling stocks will not increase the incidence of core-flush.

Comparison of results obtained from samples marked 1 over the three years shows that least core-flush was recorded in 1946, the year of earliest picking, indicating again that core-flush is subject to seasonal variation, irrespective of harvesting date.

TABLE III. NELSON DISTRICT 1946.

PERCENTAGES OF TOTAL AND COMMERCIALLY SIGNIFICANT COREFLUSH ON REMOVAL FROM STORAGE.

| Orchard. | Rootstock. | Total Apples per Sample. | Picking Date. | Storage Date. | Period of Storage. (Days). | Per cent. Total Coreflush | Per cent. Commercially Significant Coreflush. |
|---|---|---|---|---|---|---|---|
| 1 | Northern Spy | 497 | 8/4 | 11/4 | 203 | 73 | 27 |
| 16 | Northern Spy | 499 | 6/4 | 13/4 | 194 | 87 | 63 |
| | East Malling XII | 463 | 8/4 | 11/4 | 202 | 59 | 25 |
| 3 | East Malling XVI | 453 | 8/4 | 11/4 | 201 | 69 | 24 |

## CONCLUSIONS

Figures obtained from trees containing the susceptible varieties, Dunn's and Statesman, as intermediates showed no consistent increase in coreflush when compared with trees containing other intermediate varieties. Under commercial conditions differences in degree of core-flush can be expected between different orchards in any one season and from the same trees in successive seasons. There was no indication that the trees worked with any particular intermediate variety would give consistently higher or lower percentages of core-flush than trees budded directly on to the four root stocks listed. Thus in reworking existing trees to Granny Smith there is no need to consider the effect of intermediate variety on core-flush in future crops.

## ACKNOWLEDGMENTS

The author wishes to express his thanks to the numerous growers who supplied fruit samples, thus enabling this study to be made.

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# REVIEW

## FIVE HUNDRED VARIETIES OF HERBAGE AND FODDER PLANTS.

C.A.B. BULL, No. 39, 1948

In this publication there has been collated information concerning a great number of varieties of herbage and fodder plants, including cereals and root crops, usable as animal fodder. The objects have been to give in as concise a form as possible many details regarding origin, adaptation, characteristics and use of varieties in various countries, together with the possibilities of obtaining supplies. Much of the information on crop varieties used in the feeding of farm stock has been obtained from specialists in many parts of the world, and is therefore of special interest to all research and advisory workers, to Plant Improvement Stations, and to distributors of seeds where the aim is improved varieties for field use.

There have been difficulties in nomenclature and unfortunately several important countries have been unable to make their contributions, but, nevertheless, this first effort to bring under one cover all relevant data concerning a great number of varieties of fodder plants will be of interest and value to many scientists, seed merchants and possibly growers of improved varieties of seeds.

An index containing references to published information on crop varieties has been added as a supplement. L. W. G.

## COMMONWEALTH AGRICULTURAL BUREAUX

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The effects of dietary deficiencies on the reproductive performance of laboratory animals are briefly described, and data relating to the quantitative requirements for proximate principles, minerals, and vitamins during the processes of reproduction and rearing of the young are reviewed. An attempt has been made to correlate the results of such studies with the reproductive success obtained with stock diets subject to the limitations imposed by the operation of non-dietary variables which influence reproductive performances.

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This is the first printed account to deal with colonial soil conservation in detail. The main conservation methods are classified, and their effectiveness in different territories is discussed. The control of grazing and livestock management and systems of colonial agriculture in relation to soil conservation are described. Chapters are devoted to the use of machinery and of aerial-photographic surveys, and an account is given of legislation relating to soil conservation that has been enacted in the colonial empire.

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# THE METABOLISM AND TOXICITY OF CYANIDES AND CYANOGENETIC GLUCOSIDES IN SHEEP

## 1. ACTIVITY IN THE RUMEN.

By I. E. Coop and R. L. Blakley
Lincoln College, New Zealand

*Summary*

(1) A sheep with permanent rumen fistula has been used to study the production of HCN from cyanogenetic glucosides and cyanogenetic plants in the rumen and the absorption of this HCN from the rumen.

(2) Absorption of HCN from the rumen is very rapid. On an average 75 per cent of. dosed HCN is absorbed within 15 min.

(3) The hydrolysis of free glucoside and of glucoside in plant tissue in the rumen is rapid and can be complete in as short a time as 10–15 min.

(4) Hydrolysis in the rumen does not necessitate the presence of plant enzyme since the ruminal bacteria can hydrolyse cyanogenetic glucosides very rapidly.

(5) Peak production, concentration and absorption of HCN under most circumstances take place within a few minutes (less than 10 min.) of dosing free glucoside and within 10–20 min. of sheep eating cyanogenetic plant material.

(6) The conditions of the rumen affecting the rates of hydrolysis of glucosides and of absorption of HCN are discussed.

In spite of extensive literature on cyanide poisoning, whether by pure cyanides or by the ingestion of plants containing cyanogenetic glucosides, considerable speculation still exists as to the precise mechanism of this poisoning and the manner in which cyanide is metabolized by the body. The first step in poisoning by free cyanide is obviously the absorption of HCN from the alimentary tract, but in the case of poisoning following ingestion of cyanogenetic plant material this absorption must be preceded by hydrolysis of the glucoside to yield the free HCN. In ruminants HCN must be absorbed from the rumen, as indicated by the following facts. The rapidity with which animals react to free cyanide administered per os indicates that absorption must be rapid and must take place before there is any possibility of significant amounts of the material passing further down the alimentary tract. This is confirmed by analysis of the ruminal and intestinal contents of sheep poisoned by cyanide. The appearance of symptoms of cyanide poisoning following ingestion of cyanogenetic plants is somewhat delayed, being seldom earlier than 5 min. after commencement of ingestion and may even be several hours after. Even so, the material is still almost certainly in the rumen and would not have had time to pass on in significant quantities to the reticulum, omasum and abomasum. This again is confirmed by chemical analysis of stomach contents. Auld (1) and van der Walt (2).

The hydrolysis of the glucoside is carried out by enzymes, normally present in the plant along with the glucoside, and it is believed that hydrolysis commences as soon as the plant is ingested. However, some plants contain glucoside only, some enzyme only, and some neither, even amongst different varieties in the same species (e.g., *Trifolium repens*).

In these cases it has always been assumed (van der Walt *loc. cit.*, Seddon and King (3) ) that the plant containing glucoside only is not toxic unless another plant containing enzyme is also ingested, for otherwise the glucoside is not hydrolysed. Once released by hydrolysis the absorption of HCN can proceed in the same manner as if it had been administered as HCN or KCN. In theory the cyanogenetic plant should be as toxic as the equivalent amount of free HCN, but this has been shown by a number of investigators not to be true (see Part III). A number of factors can influence the release and absorption of HCN. The first and most important is the presence or absence of the enzyme. The second is the pH of the rumen, since under very acid or alkaline conditions the enzymic hydrolysis is inhibited or delayed (Auld, *loc. cit.*). In addition, under alkaline conditions, especially when chalk or lime is included in the diet, it has been postulated that HCN occurs as cyanide ions which cannot be absorbed through the ruminal wall (van der Walt, *loc. cit.*). Obviously other factors must also exert some influence, e.g., the physical state of the ingesta and ruminal contents and the nature of the previous diet.

In order to determine the part that all these factors play in the toxicity of cyanogenetic plants and to provide data for the interpretation of the result of dosing and feeding trials with free HCN, glucoside and with cyanogenetic plant material it was decided to investigate the fate of cyanide and cyanogenetic glucosides in the rumen of sheep. To this end a rumen-fistulated sheep was used for measuring the changes taking place in the rumen after introduction of free HCN and free glucoside into the rumen, and after ingestion of white clover, dried powdered white clover and linseed meal.

## Experimental Material and Methods

The sheep with the rumen fistula was a typical three-year-old Romney wether. The operation was performed in August, 1946, according to the technique of Phillipson and Innes (4).

The cannula was made of perspex turned down so that an outer flange could be screwed down on to an inner flange within the rumen, thus holding in contact the wall of the rumen and the abdominal wall. The external end of the cannula tube was sealed by a cap screwing down on to the cannula. The dimensions of the cannula were: Length 6.5 cm., diameter of flanges 3.0 cm., external diameter of shaft 2.1 cm., internal diameter of shaft 1.6 cm.

During the year following the operation the sheep was used for experiments in digestion. In August, 1947, it was noticed that a complete seal which had been made between the ruminal wall and the abdominal wall was beginning to grow over the inner end of the cannula. The cannula came away easily, leaving an opening fistula. From then onwards a glass rod 11 cm. long and 1.0 cm. diameter with a flange at the outer end was used to maintain the fistula and to make it air-tight. The rod was held in position both by the fistula itself and by a string passed round the sheep and tied to the flange on the rod. The work to be described took place from September, 1947 to May, 1948. During this period the sheep was housed indoors except for occasional periods of a few days out on pasture. Its ration was chaffed lucerne hay plus chaffed timothy hay. At all times it remained in good health and vigour, increasing in weight from 140 lb. to 164 lb. over the period.

*Potassium Cyanide*

HCN was administered as KCN. The pH and buffering power of the rumen was such that this is equivalent to dosing with HCN. The KCN employed was usually in the form of a 0.1 M. solution (= 2.7 mg. HCN/ml).

*Glucoside*

Lotaustralin, the cyanogenetic glucoside of white clover, was used. The sample used was a crystalline solid, 90 per cent. pure, containing 90 mg. HCN per g. Lotaustralin is very stable to acid and alkali. Boiling with 2N. acid or alkali for 15 minutes produces less than 1 per cent. hydrolysis. Consequently there can be no hydrolysis of the glucoside by acid or alkali under the conditions of experimentation to be described.

*Enzyme*

Linamarase, the enzyme hydrolysing lotaustralin, and linamarin was prepared from linseed by the method described by Coop (5) taking it as far as 'Preparation I'.

*pH Measurement*

A glass electrode pH meter made by the Cambridge Instrument Co. was used.

*Determination of HCN*

It was essential in this work to use a method of analysis which was rapid, sensitive, and specific. The only method which reasonably fulfils these three requirements is that devised by Gettler and Goldbaum (6). This relies on the Prussian Blue test, which is developed by aeration of the HCN solution and passage of the gases through filter paper impregnated with ferrous sulphate and sodium hydroxide. After aeration the excess of iron hydroxide is dissolved out with hydrochloric acid. Standard Prussian blue stains are prepared with which to compare the unknowns. The method is specific and no known substances interfere with it. It is specific and no known substances interfere with it. It is also rapid as the aeration takes only five minutes. This is an important advantage over the methods such as that of van der Walt and other investigators, for where samples are being taken at intervals of every 10-20 minutes a reaction can be followed as it proceeds, since a rough estimate of the HCN value can be made immediately after placing the test paper in the acid. At a later stage one can analyse the duplicate sample and estimate all the values accurately by comparison of the stains with the standards. As regards sensitivity the method as used in this study covers the range 0.5–20.0 μg. (micrograms). Gettler and Goldbaum claim an accuracy of 0.1 μg. within this range, but this accuracy was seldom achieved in our work. There was often a considerable variation (up to 15 per cent.) between duplicate stains, and in this case it was necessary to make three estimations per sample. The deepest stain was in all cases taken as the correct one, since estimations of standard solutions showed negative errors only. With these precautions it is estimated that the error is of the order of 10 per cent. when the estimation is done in duplicate and 5–10 per cent. when done in quadruplicate.

Estimations on pure KCN solutions at room temperature were subject to errors of the magnitude described. These must be attributed to irregularities in the test papers and to errors in reading. Gettler and Goldbaum dried the test papers, both after ferrous sulphate and after sodium hydroxide impregnation, for an unspecified period in air. Our own experience showed that more satisfactory results are obtained by drying the papers at 60–70°C. by suspending them in air vertically over a hot plate until thoroughly dry. This avoids both the formation of a hard crust of sodium carbonate, which makes it difficult to draw the gases through the paper, and too much oxidation to the ferric state. For the same reason the papers should be stored immediately in an air-tight vessel, where they may be kept for about a week. Using either procedure for drying the resulting papers are far from uniform, and irregularities in drying, oxidation, pore size, and carbonate formation must account for much of the error of the method.

Even if these errors could be eliminated, it is not possible to compare two stains by the unaided eye with an accuracy greater than 5 per cent. Hence the errors inherent in the method preclude an accuracy greater than this.

A further improvement was effected by replacing the rubber bands in the apparatus of Gettler and Goldbaum by perspex clamps. This is an important point, since if the glass flanges are not pressed tightly and uniformly together the blue stain forms in one small region of the paper. After a few days the test paper becomes coated with carbonate, making it difficult to draw air through the paper, and any small leakage of air causes bad distribution of colour over the paper.

### METHOD OF DOSING AND SAMPLING

The apparatus used consisted of a piece of glass tubing about 25 cm. long and 1 cm. external diameter attached by means of a short piece of pressure tubing to a splash head bulb which in turn was attached to a 100 ml. syringe. The solution to be dosed was sucked up into the bulb. The rod plugging the fistula was then removed, the 25 cm. tube introduced into the rumen, and the liquid injected by closing the syringe. Immediately after withdrawing the tube the fistula was plugged again. In some cases the solution was given through the fistula by means of a drenching gun.

At varying intervals after giving the dose, samples were taken from the rumen. This was performed, using the above apparatus, by introducing the tube into the rumen, sucking the fluid slowly into the bulb, and then forcing it into a sampling tube. Two separate samples were taken each time. Since the dose and the sample were administered and withdrawn through the same opening (fistula) and with the same apparatus, it was necessary to exercise considerable care after dosing with KCN to avoid contamination of the sample.

It was often difficult to obtain a sample if the sheep had just previously eaten to capacity, especially on the chaffed hay usually provided. In such an event it was necessary to wait for the rumen to contract, when any fluid from the rumen and reticulum was thrown up on top of the solid ruminal mass. Such a sample was not a true sample of the rumen, but was representative of the fluids draining and circulating through the rumen. Because of this difficulty most experiments were conducted while the rumen was reasonably fluid.

If the sheep was fed in the evening the rumen was usually in a satisfactory state next morning and a light feed could even be given before and during the experiment.

The sample tubes were 15 ml. centrifuge tubes, into each of which 1 ml. of 0.5 N. NaOH had been introduced. The sample of ruminal fluid varying from 5–12 ml. was then added from the sampling apparatus. The tubes were immediately corked and shaken. The NaOH by making the fluid alkaline (pH 9–10) prevented loss of HCN and also, after glucoside dosing, any hydrolysis of the glucoside and consequent increase in HCN after sampling. Thorough tests showed that this technique was effective and that within experimental error no loss of HCN occurred. The level of the fluid in each tube was then marked so that the volume of sample taken could subsequently be measured. HCN estimations were then made on these samples. Where the HCN content was relatively high the sample was diluted to bring it within the range of estimation. Before an aliquot was taken from a diluted sample it was necessary to acidify because the NaOH makes the ruminal fluid so mucilaginous that it does not disperse evenly, even when shaken with water.

## Results

### *Rate of Absorption of HCN from Rumen*

The rate of absorption of HCN, dosed as KCN, was measured by the technique described. The assumption is made that the disappearance of HCN from the rumen is due to absorption from the rumen and to no other cause. The disappearance is so rapid that it cannot be due to passage down the intestinal tract or to detoxication within the rumen (see Part II). The results obtained are set out in Table I.

Table I. Concentration of HCN in Rumen at Intervals After Dosing, Concentration Expressed as μg./ml.

| Experiment No. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| Dose (as HCN) | 40 mg. | 72 mg. | 72 mg. | 97 mg. | 100 mg. | 110 mg. | 128 mg. | 150 mg. |
| pH of rumen | 5.54 | 8.45 | 6 76 | — | 8.02 | 6.62 | 6.62 | 5.52 |
| Time (minutes) | | | | | | | | |
| 0 | (13) | (24) | (24) | (30) | (33) | (36) | (43) | (50) |
| 5 | — | 8.7 | 6.7 | 10.2 | — | — | — | — |
| 15 | 3.9 | 4.8 | 2.2 | 1.8 | 7.8 | 8.6 | 8.5 | 25 |
| 30 | 2.9 | 3.0 | 1.7 | 0.5 | 3.5 | 2.5 | 1.2 | 4.3 |
| 45 | 2.3 | 1.5 | 0.9 | 0.0 | 2.4 | 1.3 | 0.8 | 3.2 |
| 60 | 1.9 | | | | 1.8 | | | 2.5 |
| 120 | 0.8 | | | | 1.4 | | | 1.3 |
| 180 | 0.1 | | | | | | | 0.7 |

The concentrations at 0 minutes are the theoretical initial values, assuming the volume of the rumen to be 3 litres and assuming complete mixing. The concentrations at the intervals stated were those measured. Sometimes the time intervals were not strictly those given, in which case the concentrations were obtained by interpolation, but in no case was the smoothing of curves done. It was found that concentrations measured between 0–10 min. were unreliable in that considerable variations existed between duplicate samples. This was undoubtedly due to incomplete distribution of the dosed cyanide throughout the rumen, and the resultant possibility of striking a pocket of high HCN concentration. As a result the first sample was usually taken 15 min. after dosing.

The experiments took place at various times over a period of eight months. With the exception of Experiments 2 and 5, the sheep had been fed normally and had access to food during the experiments. In the case of Experiments 7 and 8, fairly severe symptoms of cyanide poisoning were manifest. In order to test whether either starvation or a high pH affected the rate of absorption, the sheep was starved for 48 hours before dosing in Experiments 2 and 5, whereas in the other experiments it had been dosed immediately after a full feed. Several observations emerge from these results:—

(i) The rate of absorption of HCN is extremely rapid. On an average, about 75 per cent. is absorbed within the first 15 min. and over 90 per cent. within half an hour of dosing.

(ii) A variation between experiments, though not great, does exist and cannot be attributed with reliability to any definite cause. There is no significant correlation with pH or degree of feeding. Part of it is probably due to experimental error in sampling and estimation, but it is unlikely that this would account for more than a small fraction of the total.

(iii) The effect of starvation and alkaline conditions (Experiments 2 and 5) in the rumen does possibly reduce the rate of absorption slightly, but it is within the normal variation of normal sheep. Certainly the effect, if any, is not marked.

(iv) The rate of disappearance of HCN from the rumen is so rapid that absorption directly from the rumen must be the means of removal.

(v) Owing to the variation between experiments, it is difficult to estimate a true rate of absorption. However, taking the average of the whole eight experiments, the rate of absorption in the first 15 min. at an average concentration of 20 μg./ml. is about 1.7 μg./ml. per minute. Over the period from 15–45 min. at an average concentration of 3–4 μg./ml. the rate of absorption is about 0.1–0.15 μg./ml. per min.

*The Hydrolysis of Cyanogenetic Glucosides by ruminal microflora*

Before studying the effect of dosing with glucoside, it was shown that microflora of the rumen could hydrolyse the glucoside with release of HCN. Preliminary experiments of introducing lotaustralin into the rumen showed that HCN could be detected in the ruminal contents several minutes after dosing. This could be due to HCN already present in the diet, to enzyme present in the diet, or to bacterial hydrolysis of the glucoside. Experiments showed that the lucerne hay which formed part of the diet did contain extremely small amounts of both cyanogenetic glucoside (0.5 μg. HCN/g.) and enzyme, but such amounts were quite incapable of accounting for the rate of hydrolysis of the lotaustralin. The diet of the sheep was nevertheless changed to one of turnips, chou moellier, and crushed oats, all of which were shown *in vitro* to be totally devoid of either glucoside or enzyme. After a week on this diet the results were precisely the same.

Ruminal liquor was then taken and its activity determined by its rate of hydrolysis of lotaustralin *in vitro* (in Conway units). Some of the liquor was at the same time centrifuged. Centrifuging at 500 r.p.m. for 1½ min. threw down material containing most of the activity while the supernatant liquid after centrifuging at 2,500 r.p.m. for 10 min. contained only a small percentage of the original activity.

Finally cultures of the ruminal microflora were made in liquid and solid media containing tryptose and lotaustralin. Two organisms were found to grow well under aerobic conditions: a Gram positive diplococcus, and a Gram negative bacillus. Cultures of these organisms readily hydrolysed lotaustralin. These facts, together with the data to be presented in the remainder of this paper, leave no doubt that the ruminal microflora are capable of releasing HCN from cyanogenetic glucosides.

*The Rate of Hydrolysis of Lotaustralin by ruminal microflora in vitro*

The rate of hydrolysis of lotaustralin by ruminal microflora was measured by withdrawing a sample of rumen liquor and rapidly transferring this to an incubator where any sediment and large food particles were allowed to settle. The supernatant liquid was decanted off into the reaction tubes. Glucoside was then added at a rate varying from $\frac{1}{2}$–1 ml. of solution per 10 ml. of ruminal liquor. The time interval between taking the sample and the commencement of the reaction was less than 5 min. The temperature was maintained at 37°c. The reaction vessel was a test-tube, tightly corked, from which 1 ml. samples were taken very rapidly at intervals for analysis. At first the incubation was carried out under a layer of paraffin oil to prevent loss of HCN and to maintain an aerobic condition, but this proved to be unnecessary. The simple technique described might be criticized on the grounds that there would be loss of HCN each time the tube was opened and a sample taken. Using glucoside of known strength it was found that within experimental error (± 5 per cent.) there was no loss. The reaction is certainly aerobic, whereas in the rumen it is anaerobic, but this likewise had no significant effect, possibly because the reaction is fast and completed before aerobic conditions have time to modify the bacterial population and activity. In the interests of rapid working this crude but effective method was used in all incubation experiments.

Some typical results obtained are given in Table II:—

Table II. Rate of Hydrolysis of Lotaustralin

| Date. | Concn. of Glucoside as HCN in Reaction Vessel. (μg./ml.) | pH. | Time to 50 per cent. Hydrolysis. (Minutes) |
|---|---|---|---|
| 22/9/47 | 17 | 6.8 | 16 |
| 24/9/47 | 17 | — | 22 |
| 27/4/48 | 30 | 6.0 | 10 |
| 5/5/48 | 30 | 7.4 | 10 |
| 18/8/48 | 30 | 7.3 | 4 |

These rates show that the hydrolysis is rapid. In the sheep itself the rate must be even more rapid, since in the *in vitro* work there is dilution of about 10 per cent. and some chilling (about 7°c.) in the few minutes following withdrawal of the sample. The concentrations of glucoside in these reactions are the same order as those used in the subsequent *in vivo* experiments. The results indicate that the maximum rate of production of HCN should occur at an interval not greater than about 15 min after administration of glucoside.

The optimum pH of the bacterial reaction was measured by the same technique. The sample withdrawn was brought to the desired pH by the addition of either dilute acetic acid or caustic soda. The rates of hydrolysis were then measured and corrected for the slight dilution by acid or alkali. This was repeated at a subsequent date, but the two determinations agreed well. The pH curve so obtained is given in Fig. 1.

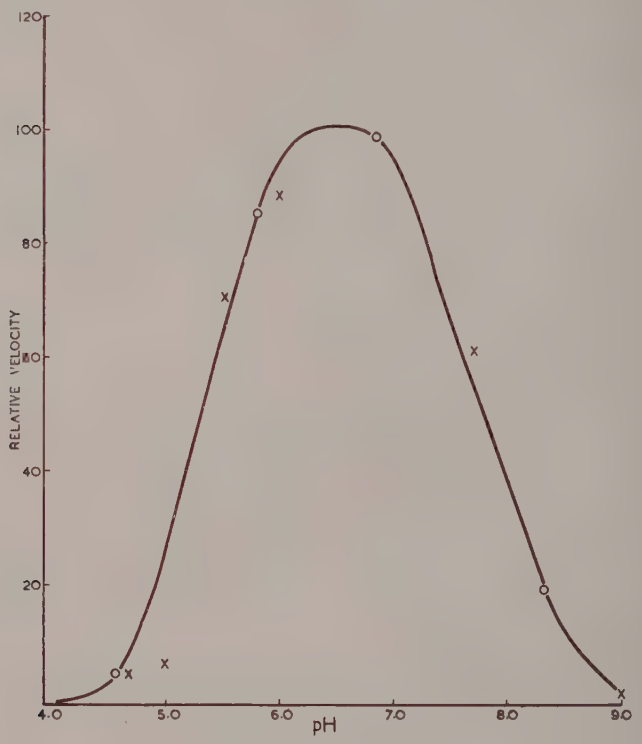


FIG. 1

By comparison with the pH curve of the enzyme linamarase (Coop, *loc. cit.*) the bacterial hydrolysis is much less tolerant to acid conditions. Whereas linamarase possesses 90 per cent. of its maximum activity at pH 5.0 and 60 per cent. at pH 4.0 the bacterial activity is reduced to a low value even at pH 5.0. This indicates that the hydrolysis in the rumen is a function not only of the enzyme system within each bacterium, but is also a function of the medium as an environment for bacterial growth and metabolic activity. The normal range of the pH found in the rumen of sheep is approximately 5.5–7.5 which coincides with the conditions of optimum hydrolysis by ruminal fluid. On the alkaline side, the activity falls to below 10 per cent. of the maximum at a pH of 8.6. This fact will be referred to later, in Part III, for it is possible to delay the hydrolysis in the sheep by inducing an alkaline reaction in the rumen.

*The Effect of Salts and Sugar on Rate of Hydrolysis in vitro*

At various times it has been stated that the administration of glucose, sulphur, sodium thiosulphate, ferrous sulphate, and other substances is beneficial in the treatment of cyanide poisoning (van der Walt, *loc cit.*). The effect of these substances on the hydrolysis of glucosides was measured *in vitro*. 10 ml. of rumen liquor was incubated for 15 min. with each of the following substances and then 1 ml. of glucoside solution added to give a concentration of .15 mg./ml. (equivalent to 15 μg. HCN/ml.). The following results were obtained (concentrations expressed as the concentration in the reaction vessel):—

(i) Glucose 50 mg./ml. Velocity reduced to less than 5 per cent.

(ii) Glucose 10 mg./ml. Velocity reduced to approx. 10 per cent.

(iii) Glucose 2 mg./ml. Velocity reduced to approx. 50 per cent.

(iv) Sulphur (Flowers) 1 mg./ml. Velocity reduced to approx. 75 per cent.

(v) Sodium Sulphide 1 mg./ml. Velocity reduced to approx. 70 per cent.

(vi) Sodium Thiosulphate 1 mg./ml. Velocity reduced to approx. 80 per cent.

(vii) Sodium Nitrate 1 mg./ml. Velocity reduced to approx. 75 per cent.

(viii) Ferrous Sulphate (hydrated) 1 mg./ml. Velocity reduced to approx. 50 per cent.

It will be noticed that all reduced the rate of hydrolysis, the most significant being glucose. The salts and sulphur in the concentrations used certainly reduced the rate to some extent, and whether this was due to a depressing effect on bacterial activity or due to their entering into some detoxication mechanism is not known. The effect of glucose is noteworthy. A dose of 30 g. of glucose (equivalent to 10 mg./ml. in a 3-litre rumen) reduces the rate of hydrolisis to 10 per cent. of its original value. No doubt this is the cause of the antidotal value of glucose. van der Walt *(loc. cit.)* and Worden (7), explain the influence of glucose as combining with HCN to form the cyanhydrin. This is unlikely, seeing that the cyanhydrin is already an intermediate compound in the hydrolysis of glucoside to yield glucose, HCN and another constituent. A more likely explanation is that the readily available glucose competitively inhibits attack on the glucoside by the bacteria. When there is ample glucose there is no need to utilise glucoside. Further proof that glucose does not hold the HCN as cyanhydrin was obtained by aerating solutions containing 3 μg./ml. and 1 μg./ml. of HCN at 37°c. and at a pH of 6.5, alone, and in the presence of 2 per cent. glucose. The glucose did not decrease the rate at which the HCN was given off by aeration. The HCN plus glucose was then incubated for 40 min. both with and without ruminal liquor. This did not have any effect either, though incubation for 24 hours did procure a significant reduction. The conclusion must be reached therefore that glucose does not affect the absorption of HCN in the rumen but influences its rate of production from the glucoside.

*Rate of Hydrolysis of Glucoside and of Absorption of HCN in vivo*

Having shown that the microflora of the rumen can bring about the Hydrolysis of lotaustralin with release of HCN at such a rate that the addition of enzyme preparations would not be of any advantage, all administrations of glucosides were carried out in the complete absence of enzyme.

Glucoside solution was introduced into the rumen through the fistula, and ruminal samples taken at intervals for determination of HCN present. In the case of HCN administration samples taken for HCN estimation in the first 10–15 min. tended to be unreliable, but where one is measuring HCN produced from a substance containing no free HCN it would be expected that the values would be more consistent and more reliable. This was found to be true, though there were still irregularities due to incomplete mixing in the rumen.

The results obtained at various times over a period of 8 months are given in Table III. In every case the dose was the same, 1.1g. of 90 per cent. lotaustralin (equivalent to 100 mg. HCN).

TABLE III. CONCENTRATION OF HCN IN RUMEN EXPRESSED AS μG./ML. AT INTERVALS AFTER DOSING WITH LOTAUSTRALIN

| Experiment No. | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
|---|---|---|---|---|---|---|---|
| Dose (as HCN) | 100 mg. | 100 mg. | 100 mg. | 100 mg. | 100 mg. | 100 mg. | 100 mg. |
| pH of rumen | — | 6.10 | 6.32 | 7.12 | 6.54 | 6.82 | 8.12 |
| Time (Minutes) | | | | | | | |
| 5 | 6.7 | — | — | 7.7 | 20 | 6.5 | — |
| 10 | 3.1 | 9.7 | | 7.2 | 15 | — | |
| 15 | 1.3 | 3.0 | 6.1 | 4.8 | 5 | — | 6.2 |
| 30 | 0.2 | 0.6 | 3.9 | | | 3.4 | 5.0 |
| 45 | — | 0.2 | 2.7 | | | 1.9 | — |
| 60 | 0.0 | 0.1 | 2.5 | | | | 4.3 |
| 120 | | | 0.1 | | | | 3.0 |
| 180 | | | | | | | 2.1 |

The values quoted are those actually obtained, and are not taken from smoothed curves. Assuming a ruminal volume of 3 litres, the theoretical HCN concentration for complete hydrolysis and without any absorption of HCN would be 33 μg./ml. The actual concentration in the rumen is the difference between the rate of production of HCN by hydrolysis and the rate of absorption of the HCN produced. The former can vary according to the concentration and activity of the bacteria, while the latter varies according to concentration in a manner illustrated in Table I.

Several observations can be made on these figures:

(i) The rate of hydrolysis is very rapid. In the case of Experiment 13, apparent hydrolysis had proceeded to the extent of 60 per cent. within 5 min. and in Experiments 9, 12, and 14 to the extent of 20–25 per cent. The actual amount of hydrolysis would be even greater since no allowance for absorption has been made.

Excluding Experiment 15, which was abnormal (see below) hydrolysis must for all practical purposes be complete at times, varying from 10–15 min. in the case of Experiment 9 to 1 hour in Experiment 11.

(ii) Considerable variation exists in Experiments 9–14 between the concentrations at any given time. This could not be explained in terms of

pH, amount of feed eaten, and consistency of ruminal contents. The variation is probably due to varying concentrations and activities of the ruminal bacteria which in turn depend upon the distribution of food intake over the previous 24 hours. Unfortunately, the latter was not recorded at the time.

(iii) Experiment 15 was specifically designed to reduce the bacterial activity. The sheep had been starved for 60 hours at the time of dosing, the pH was high (8.12) and the ruminal contents were very fluid. In the morning before dosing, KCN had been administered to determine whether the starvation affected rate of absorption of HCN (Experiment 5, Table I), and it was found that the latter was not markedly slower than normal. The data in Experiment 15 show that the rate of hydrolysis is definitely reduced. Initially at least 20 per cent. hydrolysis must have occurred in the first 15 min. but the maintenance of a concentration of .2–3 μg./ml. at 2–3 hours shows that there is still significant hydrolysis taking place even then. The reduction in rate may be due either to a reduction in the concentration of bacteria or to pH alone or both.

*Hydrolysis of Lotaustralin in Wet Clover*

The experiments described above dealt with the glucoside in a free state. In practice it is the fate of the cyanogenetic glucosides in the plant as eaten that is of greatest interest. The sheep was therefore fed cyanogenetic plant material and the concentration of HCN in the rumen measured.. Parallel experiments were conducted *in vitro* to determine the rate of hydrolysis by ruminal fluid.

The first plant material given to the sheep was white clover. The strain used contained both glucoside and enzyme. As it was considered important to know the rate of eating the sheep was not put out on to a clover paddock, but the clover was cut and fed indoors. Details of the experiments were as follows:—

(i) Experiment 16. The clover was cut with a scythe, gathered up and fed to the sheep within 10 min. The clover itself was rather damp. The HCN content was 0.008 per cent. pH of rumen = 6.17. The sheep ate 650g. in 45 min., after which the clover was removed.

(ii) Experiment 17. The clover was dry, the day being fine and sunny. It was fed 10 min. after cutting. The sheep ate 800g. in 15 min., after which the clover was removed. HCN content was 0.008 per cent., but the pH of the rumen was not recorded.

(iii) Experiment 18. Same as for Experiment 17. Sheep ate 850g. in 15 min.

The results of these experiments are given in Fig. 2. In this graph are also plotted the results of *in vitro* experiments in which clover was incubated to determine the rate of HCN production. The clover was picked from the same plot, but unfortunately not on the same days as the previous *in vivo* experiments, so that the results are not strictly comparable. The clover was bruised in a mortar to stimulate the physical state of ingested clover in the rumen and was then incubated both with ruminal liquor and with water (3 g. clover+12 ml. ruminal liquor or water). The time from picking to incubation was 15 min.

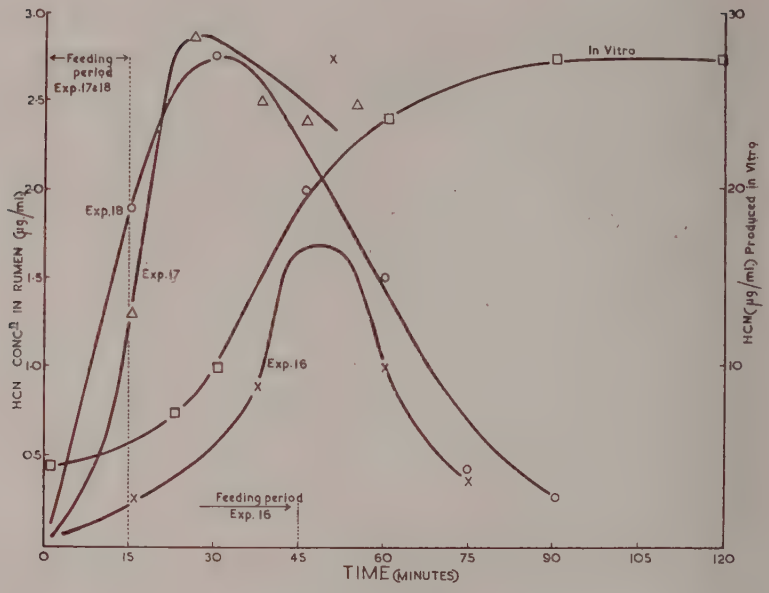


FIG. 2

The special features of these results are as follows:—

(i) The *in vitro* hydrolysis is considerably slower than that of free glucoside, the time to half decomposition being of the order of 40 min. as against 10–20 min. for free glucoside. Furthermore, the enzyme present in the clover must account for most of the HCN released, even in the presence of ruminal liquor, since addition of the latter did not accelerate the production of HCN.

(ii) Though the eating of 800 g. of clover is equivalent to 64 mg. HCN at no time did the HCN level of the rumen rise above 3 μg./ml. This is undoubtedly due to the slower rate of hydrolysis so that the HCN is absorbed almost as fast as it is produced.

(iii) Peak HCN concentration is reached fairly consistently about 20 min. from the mid-point in time of the feeding period. This indicates that the *in vitro* reaction is slower than the *in vivo,* which is to be expected.

*Hydrolysis of Lotaustralin in Dried Clover*

A sample of dried clover was made available from the Plant Chemistry Laboratory, Palmerston North. This clover contained glucoside, equivalent to 0.104 per cent. HCN, but it contained no enzyme at all. Incubation of the clover alone produced no HCN. Unfortunately, in anticipation of introducing the clover through the fistula the dried material had been ground. As it subsequently proved difficult to introduce through the narrow hole of the fistula at a sufficiently rapid rate it had to be fed to the sheep. Being a powder, it was difficult to eat, but with some training and the inclusion of 33 per cent. crushed oats, the sheep was coaxed to eat some.

(i) Experiment 19. The sheep ate 160 g. over a period of 96 min. The HCN level of the rumen remained fairly constant between 0.8–1.0 μg./ml. during the feeding and fell to less than 0.1 μg./ml. 40 min. after taking the clover away.

(ii) Experiment 20. The sheep ate 66 g. (= 69 mg. HCN) within 15 min., after which the clover was removed. pH of rumen was 6.0.

(iii) Experiment 21. The sheep ate 50 g. (= 52 mg. HCN) in 15 min. and the feed was then removed. pH was not recorded.

Simultaneously with the *in vivo* experiments, Nos. 20 and 21, *in vitro* incubations were made, using 15 ml. ruminal liquor plus 0.5 g. dried clover, this proportion being of the same order as that in the rumen. The results of Experiments 20 and 21 and of the parallel *in vitro* experiments are given in Fig. 3. As it was thought that the powdering of the dried clover might be responsible for the rapid rate of hydrolysis, the dust was sieved out and the coarse fraction not passing a 40 mesh sieve was compared with the original material. This fractionation did not affect the rate of hydrolysis, the only difference being that the coarse fraction was rather lower in HCN than the original. In Fig. 3 the *in vitro* curves are those obtained using the coarse material.

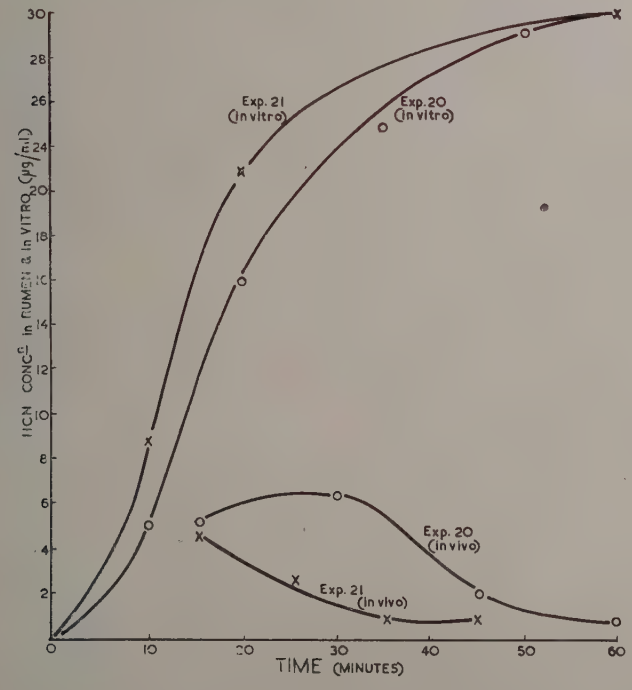


Fig. 3

From Fig. 3 it will be seen that:

(i) The time to half decomposition in the *in vitro* incubations is 17–19 min. Even in the complete absence of enzyme the bacterial hydrolysis is very rapid.

(ii) Peak HCN concentration *in vivo* is reached within 10–25 min. from the commencement of the feeding period and is reached earlier on the day in which the *in vitro* hydrolysis is more rapid (Experiment 21).

*Hydrolysis of Linamarin in Linseed Meal*

The only linseed preparation available was in the form of commercial linseed nuts, which were low in HCN content (0.030 per cent.) and were lacking in enzyme activity.

*In vitro* incubation of crushed linseed nuts gave zero HCN due to absence of active enzyme, but when incubated with ruminal liquor at the rate of 0.5 g. linseed to 15 ml. of liquor the time to half-hydrolysis was 15 min. The half-hydrolysis time, using free lotaustralin and the same ruminal liquor was 10 min.

*Experiment 22.* Simultaneously with the incubation the sheep was given uncrushed linseed nuts, eating 380 g. in 15 min., after which the linseed was removed. The HCN content of the rumen was as follows, the times quoted being from commencement of feeding:—

1.6 μg./ml. at 15 min., rising to a maximum of 2.6 μg./ml. at 30 min., and falling to 0.5 μg./ml. at 90 min. The pH was 7.4.

On another occasion it ate 622 g. in 15 min. and the HCN content of the rumen fell consistently from 1.5 μg./ml. at 15 min. to 0.3 μg./ml. at 60 min. The pH was not recorded, and on this occasion no incubation was made.

These observations prove that:—

(i) Linamarin is rapidly hydrolysed by rumen microflora;

(ii) Assuming the rate of hydrolysis of linamarin and lotaustralin to be the same, which is likely on account of their similarity in constitution, the rate of hydrolysis of linamarin in crushed linseed is about two-thirds as rapid as that of the free glucoside (half time 15:10 min.).

(iii) The HCN in the rumen remains at a low level. As the nuts fed to the sheep were uncrushed, the comparison between *in vitro* and *in vivo* cannot be carried far.

*Hydrolysis of Amygdalin in the Rumen*

By the previously described *in vitro* technique it was shown that the bacteria hydrolyse amygdalin readily, the rate of hydrolysis being about 60–70 per cent. as fast with lotaustralin. As further confirmation of this hydrolysis, the sheep was dosed per fistula with 1.7 g. amygdalin equivalent to 100 mg. HCN. The pH of the rumen was more alkaline than usual, viz., 7.62. The values of HCN concentration in the rumen were as follows:—10 min., 3.9 μg./ml.; 30 min., 1.7μg./ml.; 60 min. 0.2 μg./ml.; 90 min., 0.1 μg./ml. These figures are comparable with those obtained by a similar dose of lotaustralin (Table II).

*pH and Buffering Power of Ruminal Liquor*

One would expect the fluids of the rumen to possess considerable buffering power, and this proved to be correct. Thus the addition of 1 ml. 0.5N. NaOH to 10 ml. rumen liquor usually raised the pH to 9–10, but not beyond. The buffering power is greatly influenced by the state of the ruminal contents, the rumen of a starved sheep having a much lower power. Thus the addition of 1 ml. of 0.1N. NaOH per 10 ml. of rumen liquor changed the pH by only 0.37 in a well-fed sheep and by 2.0 in a starved sheep with very fluid ruminal contents. On one occasion when lime was added it took 2 mg. CaO/ml. to shift the pH from 6.5 to 9.5. In an endeavour to produce alkaline conditions in the rumen without administering caustic alkalis the sheep was given powdered chalk. Overnight it ate 80 g. $CaCO_3$ with its food. The following morning it ate a further 40 g. with hay and 20 g. was introduced through the fistula at 11 a.m. An hour later the pH was only 6.62.

These observations are of interest, for throughout the literature references are made to acid or alkali-producing diets having an effect on HCN liberation. As the pH is not measured and quoted it is believed that such statements can be misleading. In 18 months of measuring ruminal pH's on a variety of diets and under a variety of conditions, the extreme values obtained were 5.5 and 8.5.

Fig. 1 shows that the rate of hydrolysis of glucoside by ruminal bacteria is reduced to a low level in the region of pH 8.0–9.0. Some considerable reduction in hydrolysis can therefore be expected under practical conditions at the more alkaline pH's. On the other hand, some authors (van der Walt, *loc. cit.*) also contend that these alkaline conditions decrease the rate of absorption of HCN since some of the cyanide will be present as $CN^-$ ions. This is clearly of little sigificance since the pK for HCN is 9.14 and the pH would have to hise above 9.0 before much cyanide was present in the ionic form. This has been borne out in any case by the results quoted in Table I.

## Discussion

The practical implications of the observations made in this paper will be discussed in Part III.

## Acknowledgments

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# SHEEP DIPPING TRIALS WITH DERRIS, BENTONITE SULPHUR, D.D.T., AND BENZENE HEXACHLORIDE

By I. E. COOP and G. B. MCLEOD
Lincoln College, New Zealand

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## *Summary*

Dipping trials have been conducted with derris, bentonite-sulphur, D.D.T. and benzene hexachloride against the sheep ked and the sheep louse. Derris has been shown to be very effective against keds but its control of lice has not been satisfactory. Bentonite-sulphur has proved to be unsatisfactory for the control of both keds and lice. D.D.T. has been shown to be capable of controlling keds but, like Derris, cannot be relied upon to control lice. The best material tested was benzene hexachloride, which controlled keds with the same efficiency as derris or D.D.T., but in addition, as far as the experiments have gone, has shown promise of controlling lice. The practical limitations and possibilities of the use of these materials in sheep dipping are discussed.

## INTRODUCTION

In recent years attempts have been made to produce sheep dips other than the standard arsenic and phenolic types. The latter are far from ideal in that they are poisonous and are not completely effective. Whilst, for instance, the majority kill all adult keds at dipping, the materials do not last long enough in the fleece to kill the pupae when they hatch, and so provide little measure of control. Considerable improvement in effectiveness was made when the so-called 'quick-acting' types came into use. In these an extract of Derris root, containing the active principle, rotenone, was incorporated and this gave a greater measure of ked control for it killed all adults and remained in the fleece for sufficient time to be toxic to newly hatched pupae. Even so, these commercial dips are not entirely satisfactory. They are poisonous to sheep and humans, they are expensive and their period of toxicity in the fleece is short. Thus any dip which is non-poisonous, is cheap and confers a longer period of toxicity, is to be preferred. In the ideal this period of persistence of toxicity needs to be of the order of 12 months, so that reinfestation from 'stragglers' or unmustered sheep cannot take place from one shearing or dipping until the next, one year later. In such an event external parasites should be capable of eradication with comparative ease even on our rough hill country.

In 1941 Morrison and McLeod (1) first suggested the use of derris powder alone as a dipping material for the control of keds (*Melophagus ovinus*) and body lice (*Bovicola ovis*). Their experiments showed that a suspension of derris in water was highly toxic to keds, killing all live ones and remaining in the fleece long enough to kill all young keds emerging from pupae. Thus a single dipping was sufficient to eradicate keds completely from a flock provided it could be isolated from undipped sheep.

Though the period of persistence of toxicity was not long (of the order of 4-6 weeks) the dip had the advantage of being harmless to sheep and humans and of being very cheap. At the time it was thought that derris was also equally effective against lice, though the number of experiments on lice was fewer. Since the publication of the original work many thousands of sheep have been dipped in derris. The field experience of derris dipping together with further experimental work

to be described later, has indicated two short-comings of derris powder dips. Firstly, in a considerable number of cases, a temporary lameness has been reported by McLean (2) following dipping in derris. Secondly, our own work has subsequently shown that derris, while killing all adult lice, cannot be relied upon to control them completely. This present paper reports the results of further work with derris, particularly against lice.

In search of new dipping materials, reports from U.S.A. (3) indicated that bentonite-sulphur was toxic to keds and lice and furthermore gave a much longer period of toxicity in the fleece than any material so far examined. It was claimed that this period was about 300 days. It was decided to test these claims.

In 1946 the new synthetic insecticides D.D.T. and benzene hexachloride became available in sufficient amounts to test their effect on keds and lice. In the meantime experiments with D.D.T. and benzene hexachloride have been reported from overseas. Heath (4) showed that D.D.T. at a concentration of 0.5 per cent. in the dip killed all the keds and prevented reinfestation for a period of at least five weeks. Mitchell and Heath (5) tested D.D.T. against the sheep tick (*Ixodes ricinus*) and found it to be fairly toxic though not completely satisfactory. They also prepared and tested many different types of D.D.T. emulsion. Later, Heath (6) tried lower concentrations of both D.D.T. and benzene hexachloride against keds, the concentrations of the crude insecticides being 0.5 per cent., 0.25 per cent. and 0.125 per cent. The initial kill was complete in all cases and the period of toxicity was determined by subjecting the sheep to reinfestation. The benzene hexachloride dips remained toxic until the conclusion of the benzene hexachloride experiments 10 weeks after dipping. The D.D.T. dips were toxic 8 weeks after dipping but were no longer toxic in the fleece 14 weeks after dipping. Heath concludes that either D.D.T. or benzene hexachloride at 0.125 per cent. concentration is sufficient to eradicate keds provided isolation can be maintained. In U.S.A., Rude and Parish (7) dipped 5,000 sheep and goats in a 0.2 per cent. D.D.T. dip and showed that they remained free of keds for 13 weeks after dipping. In Australia D.D.T. has given promising results (private communication N.P.H. Graham) where good control of keds was achieved with 0.1 per cent. D.D.T. The concentration fell considerably as the sheep were put through the dip. With a 1,000 gal. power spray dip the concentration fell from 0.08 per cent. to 0.05 after 1,000 sheep had been dipped. Nicol (8) obtained complete control of keds at a concentration of 0.2 per cent. and there was also some ' stripping out ' of the D.D.T.

In the dipping trials reported in this present paper much lower concentrations of D.D.T. and benzene hexachloride were used and their effectiveness against both keds and lice was investigated. No attempt has been made to determine the effect of these dips in blowfly strike prevention, though it is recognized that this feature may also be of importance.

## Experimental Technique

The technique used was the same as that of Morrison and McLeod (1). The sheep were dipped individually in a small portable tank holding 50 gal. of dip, the sheep being held in the bath for 30 seconds approximately. After dipping, the sheep of each group were isolated in double-fenced pens. Keds and lice were counted before dipping, about 5-10

days after dipping and thereafter at monthly intervals. Keds and pupae were easy to count but lice were often difficult to find. At least 20 minutes' examination of each sheep was made before declaring it free of lice.

In recording the degree of infestation an arbitrary classification has been made into heavy, medium and light when the ked or louse populations were >30, 10-30 and 1-10 respectively. These have been recorded in the tables as +++, ++ and + respectively whilst complete absence is designated thus 0. The sheep used were sometimes of mixed breeds and type. In as far as breed type influences dipping, this has been recorded in the tables as CW—coarse woolled, such as Romney, English Leicester and Border Leicester, and as FW—fine woolled such as Corriedale and halfbred. It was recognized that the length of wool on the sheep at dipping influences the amount of dip retained by the fleece and may therefore influence the effectiveness of the dip. Consequently wherever possible, and this was in the majority of cases, the sheep were dipped with 1-3 in. of wool as would be the case under ordinary farming conditions. In a few cases sheep with 6-8 in. of wool were dipped, and this fact has been taken into account in the interpretation of results. In the large scale trials sheep were dipped under ordinary farm conditions.

## RESULTS

*Derris :*

The data relating to the effect of derris on lice are given in Table I. The observation of Morrison and McLeod that derris produces a 100 per cent. initial kill was confirmed but it was found that after many months lice sometimes suddenly reappear. In Trials 1, 4 and 5 the strength of the dip was the usual ½ lb. derris per 100 gal. In trials 2 and 3 the concentration of derris was increased to the order of 2 lb. 100 gal. in the hope that a stronger dip would prevent the reappearance of lice. The sheep of Trial 3 were housed indoors, as they were part of a different experiment and in the 12 months up to the time of removal no lice appeared. In Trial 2, however, the sheep were free of lice for nearly a year but they did reappear eventually.

Another series of trials was conducted to determine the rate of deterioration of derris. Derris, as a horticultural insecticide, is known to deteriorate on continued exposure to light and air. Similarly in the fleece its effect diminishes either due to mechanical loss of the derris particles or to destruction of rotenone. To obtain some idea of the rate of destruction of rotenone a sample of derris was spread thinly over calico sheets in a well lighted room so that any deterioration due to air and light would be accelerated. At intervals, samples were taken for determination of rotenone content by chemical analysis and of biological activity by their effect on keds. The results are recorded in Table II. All lambs and hoggets were unshorn. It will be observed that over a period of 5 months the rotenone content fell from 4.2 per cent. to 3.1 per cent. but the toxicity to keds still remained at a high level though its effect on lice was poor.

As a confirmation of the extreme toxicity of rotenone to keds, an extract of derris containing rotenone was tested under ordinary farm conditions, by dipping 260 crossbred ewes and 40 Downcross lambs. The dip contained the rotenone in emulsion form at a concentration of .01 per cent. The keds were completely eradicated. In a small

TABLE 1. DERRIS

| Trial No. | Date of Dipping. | Sheep. | Louse Population. | Wt./100 gal. (lb.) | Per cent. Rotenone in Derris. | Louse Population. Months after Dipping. 0 | 3 | 6 | 9 | 12 | 18 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 8/11/43 | 3 CW ewes | ++ | ½ | 7 | — | +(1) | | | | |
| | ,, | ,, | ++ | ,, | ,, | — | — | — | +(1) | | |
| | 18/2/44 | ,, | + | ,, | ,, | — | — | +(1) | | | |
| | ,, | ,, | + | ,, | ,, | — | — | +(3) | | | |
| 2 | 26/9/44 | 3 CW ewes | ++ | 2 | 8.3 | — | — | — | — | — | +(1) |
| | ,, | ,, | ++ | 1-1/3 | ,, | — | — | — | — | +(2) | |
| | ,, | ,, | ++ | 2-2/3 | ,, | — | — | — | — | +(1) | |
| | ,, | 2 CW ewes | +++ | Control | Not dipped | +++ | + | + | +++ | +++ | |
| 3 | 5/4/45 | 20 CW ewes<br>20 FW ewes | ++ | 2 | 5 | — | — | — | — | | |
| 4 | 20/6/46 | 9 CW ewes | ++ | ½ | 4 | — | + | | | | |
| 5 | 20/6/46 | 9 CW lambs | ++ | ½ | 4 | — | + | | | | |

NOTE: (i) The number in brackets after the + sign, thus + (1), indicates the number of sheep in the group on which lice reappeared.
(ii) Sheep were examined monthly but results are tabulated tri-monthly.

TABLE II. DERRIS, ½ LB./100 GAL.

| Date. | Sheep. | Derris Exposure. (Days) | Rotenone. (Per cent.) | Before Dipping. | | 3 Weeks and After. | | 4 Months and After | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Keds. | Lice. | Keds. | Lice. | Keds. | Lice. |
| 6/11/45 | 28 hoggets | 0 | 4.2 | + | | — | — | | |
| 23/11/45 | 2 hoggets | 17 | 4.2 | + | | — | — | | |
| 14/1/46 | 3 lambs | 69 | 3.9 | + | | | | | |
| 20/6/46 | 3 lambs | 155 | 3.1 | + | + | | (1) | | (1) |
| 20/6/46 | 3 lambs | 0 | 4.2 | + | + | | | | |
| 20/6/46 | 3 lambs | 155 | 3.1 | + | + | | + | + | + |

scale trial using the same material the fleece remained toxic to keds for 4 months. The concentration of derris recommended by Morrison and McLeod and used in the field was ½ lb. per 100 gal. At 5 per cent. rotenone content this is equivalent to a concentration of rotenone of 0.0025 per cent. At this strength complete control of keds was achieved and the period of toxicity was 4-5 weeks. Trials were made to determine the minimum concentration required to control keds, with the following results :—

(i) 0.0016 per cent. rotenone (½ lb. of 3.3 per cent. derris per 100 gal.) complete control. Toxic at 3 weeks but broke down at 6 weeks.

(ii) 0.00066 per cent. rotenone (one-fifth lb. of 3.3 per cent. derris per 100 gal.) complete control but fleece was not 100 per cent. toxic to keds introduced 3 weeks after dipping.

(iii) 0.00025 per cent. rotenone (one-thirteenth lb. of 3.3 per cent. derris per 100 gal.) incomplete control. Some pupae hatched and survived. These confirm the original observations regarding the use of ½ lb. derris per 100 gal. and indicate that it would be unwise to recommend a lower concentration if a reasonable margin of safety is required.

*Bentonite-Sulphur :*

The composition of the American material was stated to be 70 per cent. bentonite and 30 per cent. sulphur and was prepared by fusing the sulphur with bentonite and then grinding to a fine powder after it had cooled. The finer details of the method of preparation were not available. The material used here was made as follows :— 5 lb. of bentonite was thoroughly dried in an air oven and ground in a 12 in. ball mill for 3 hours, 2½ lb. of sulphur were added, the mixture ground for 8 hours, then heated to 160°C. for 1 hour, cooled and reground for 12 hours. A sieve analysis showed that 96 per cent. passed a 100 mesh and 87 per cent. passed a 200 mesh.

In 1940 trials were carried out with this material on 13 sheep heavily infested with keds. Dip concentrations were varied from 15 lb. to 60 lb. per 100 gal. In no case was a 100 per cent. initial kill produced and many keds survived the dipping. Similar results were obtained again in 1945 when 8 lambs were dipped in concentrations varying from 5-10 lb. per 100 gal.

Jet-pulverised bentonite-sulphur plus 2½ per cent. of sulphite lye was then tried. Some keds survived the dipping but within 4 months all were dead. On the other hand a similar dip a few months later failed to control either keds or lice. To test the period of protection 450 ewe lambs were dipped under practical farming conditions in a bentonite-sulphur (7 lb.) plus derris (½ lb.) mixture. The initial kill was 100 per cent. probably due to the derris. After 7 months' isolation the lambs became mixed with ked infested ewes. At mustering 10 months after dipping the lambs were lightly infested. By 1946 some of the American material was available but it contained rotenone. It was therefore difficult to separate the effects of the two components. At a concentration of 8 lb. per 100 gal. there was a 100 per cent. kill of keds but some lice survived though they too disappeared after 2 months.

The results with bentonite-sulphur have therefore been disappointing. Not only is a 100 per cent. initial kill not obtained but the claims of long protection have not been substantiated.

TABLE III. D.D.T.

| Material. | Date of Dipping. | Sheep. | D.D.T. Concn in Bath. (Per cent.) | Parasite Population. | | Ked Control. Incomplete + Complete — | Louse Population. Months after Dipping. | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Keds. | Lice. | | 0 | 1 | 2 | 3 | 4 |
| D.D.T.-Talc | 25/2/46 | 1 lamb | .045 | ++ | — | — | | | | | |
| ,, | 25/2/46 | 2 CW ewes | .045 | — | ++ | | — | — | — | — | |
| ,, | 16/7/46 | 2 CW ewes | .03 | + | +++ | — | + | | | | |
| D.D.T.-Sulphur | 16/4/46 | 1 lamb | .02 | +++ | — | — | | | | | |
| ,, | ,, | 1 CW ewe | .02 | — | + | | — | — | + | | |
| Sulphur only | ,, | 1 lamb | — | +++ | — | | | | | | |
| D.D.T.-Derris | 22/11/46 | 1 FW ewe | .02 | ++ | — | — | — | | | | |
| ,, | ,, | 1 FW ewe | .02 | ++ | +++ | — | — | — | — | | |
| ,, | ,, | 1 CW ewe | .02 | — | +++ | | — | — | + | | |
| D.D.T. Emulsion | 22/11/46 | 1 FW ewe | .1 | + | — | — | — | | | | |
| ,, | ,, | 1 FW ewe | .1 | + | + | — | — | — | + | | |
| ,, | ,, | 1 CW ewe | .1 | — | +++ | | — | — | + | | |
| ,, | 26/2/47 | 3 FW wethers | .01 | + | +++ | + | | +(2) | | | |

*D.D.T.:*

The D.D.T. dip was prepared in a number of forms. All D.D.T. concentrations are quoted in terms of the pure compound.

(1) D.D.T.-talc. The D.D.T. was dissolved in light naptha and acetone, mixed with talc, the solvent evaporated off and the D.D.T.-talc pulverized. The powder was 20 per cent. pure D.D.T., 80 per cent. talc.

(2) D.D.T.-sulphur. This is a commercial preparation containing 20 per cent. D.D.T. and 80 per cent. wettable sulphur, jet pulverized. The D.D.T. in this case was 50 per cent. pure.

(3) D.D.T.-derris. Equal parts by weight of pure D.D.T. and derris powder were ground together thoroughly in a mill. The D.D.T. particles adhered to the derris and were dispersed in water with slightly greater difficulty than derris alone.

(4) D.D.T. emulsion. This was made according to the recommendation of Mitchell and Heath. Pure D.D.T. was dissolved in benzene and emulsified in water using sodium oleate and casein as emulsifying agents on 22/11/46 and sulphonated castor oil on 26/2/46. The concentrated emulsion (10 per cent. D.D.T.) was then diluted by pouring into the water of the dip. The emulsion was stable, with particle size about 2-3$\mu$.

The results are set out in Table III. In the action on keds complete control was achieved in all cases except the .01 per cent. emulsion on 26/2/47. This was the lowest concentration reached and it seems therefore that the limiting concentration is of this order. The effect of D.D.T. on lice was not satisfactory, for while in all cases the initial kill was 100 per cent. the lice began to reappear after 2-3 months.

A small scale test on the period of persistence of toxicity of D.D.T. showed it to be 6 weeks in the case of D.D.T.-sulphur at a concentration of .02 per cent. and 3 months in the case of 0.1 per cent. D.D.T. emulsion.

*Benzene hexachloride:*

The benzene hexachloride used was the crude commercial preparation containing 12-13 per cent. of the $\gamma$-isomer. The concentrations used were of the order 0.00030 per cent.-0.0125 per cent. All concentrations throughout this work are expressed in terms of the $\gamma$-isomer. The benzene hexachloride dips were also prepared in a variety of forms:—

(1) Emulsions form. The dip used on 12/7/46 was a commercial dip in which the benzene hexachloride had been dissolved in phenol before emulsification. In that used on 22/11/46, the benzene hexachloride was dissolved in benzene and emulsified with sodium oleate and casein in the same way as the D.D.T. emulsion. The dip used on 26/2/47 was similar except that the emulsifying agent used was sulphonated castor oil. In these emulsions the majority of the particles were of the order of 2-3$\mu$ diameter, though some were considerably larger, up to 30$\mu$. This is partly accounted for by the fact that a fraction of the benzene hexachloride is not soluble in benzene.

(2) Benzene-hexachloride—derris powder. As with D.D.T. an attempt was made to absorb the benzene hexachloride on the derris particles, relying on the latter to produce the suspension. The stickiness of the benzene hexachloride made the grinding together difficult. Nevertheless a suspension was made containing particles distributed throughout the whole range 1-30$\mu$, and these sedimented rather more rapidly than derris alone. The dispersible benzene-hexachloride—derris mixture was somewhat better in physical properties.

(3) Dispersible powder. In this preparation, which was a commercial one, the benzene hexachloride had been absorbed on a solid dispersing agent, there being equal parts of benzene hexachloride and dispersing agent. This powder dispersed well. The particle size varied, most particles being in the range 1-8$\mu$ though some were as large as 15-20$\mu$ and these tended to settle out.

The results with these dips are set out in Table IV.

Except in the extremely dilute dips, complete control of keds was achieved. The dipping of 100 ewes and 100 lambs on 4/2/47 was carried out on a farm under practical farming conditions. The ewes were newly shorn and very lightly infested with keds but the lambs were unshorn and were all very heavily infested. The ewes were put through the dip first and the lambs last. They were examined 8 days later and again 67 days after dipping. No live keds were found at any time.

The period of toxicity of benzene hexachloride in the fleece was determined approximately by putting 20-30 live keds on to the sheep at various intervals after dipping and then counting the number of live and dead keds a week later. As soon as 1 ked survived it was considered that the fleece was no longer toxic. At 0.0030 per cent. (dispersible benzene hexachloride) the fleece remained toxic for 3 months; at 0.0050 per cent. (emulsion) it was toxic for 4 months and at 0.0125 per cent. it remained toxic for 5-6 months. In all these experiments concerning period of persistence of toxicity, the sheep used had 2-3 in. of wool at dipping.

The trials in the very dilute dips om 16/6/47 and 10/7/47 were designed to determine the minimum toxic concentration of benzene hexachloride. The results showed that at 0.0012 per cent. complete control was effected and that the fleece was toxic to introduced keds for a period up to 9 weeks. The 0.00060 per cent. dip also gave complete control but the fleece was not toxic to keds introduced three weeks after dipping. The 0.00030 per cent. dips, on the other hand, on both occasions failed to give complete control. A concentration of 0.0006 per cent. must therefore be regarded as the lowest limit.

The effect of benzene hexachloride on lice is less definite, because the sheep must be examined over a longer period of time. In all cases a 100 per cent. initial kill was achieved. At the time of writing lice have appeared on one sheep 5 months after dipping. In this case the dip was a derris-dispersible benzene hexachloride one which, along with the derris-benzene hexachloride mixture, was not entirely satisfactory in physical properties and was in any case at a low concentration—0.0015 per cent. In all other cases at benzene hexachloride concentrations varying from 0.003 per cent.-0.0125 per cent. no lice have yet appeared though the time since dipping varies from 6-12 months. No lice have yet appeared on the sheep dipped in the very dilute dips, 0.0003 per cent.-0.0012 per cent. though in these cases it is still only 2 months from dipping.

TABLE IV. BENZENE HEXACHLORIDE

| Material. | Date of Dipping. | Sheep. | γ-isomer (Benzene hexachloride Concn. in Bath.) (Per cent.) | Parasite Population. | | Ked Control. Incomplete + Complete | Louse Population. Months after Dipping. | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Keds. | Lice. | | 0 | 3 | 6 | 9 | 12 |
| Benzene hexachloride Emulsion | 12/7/46 | 5 lambs | .0050 | + | +++ | − | − | | | | |
| | 22/11/46 | 3 FW ewes | .0125 | + | ++ | − | | − | | | |
| | 26/2/47 | 3 FW ewes | .0030 | + | +++ | − | − | − | − | | |
| Benzene hexachloride-Derris | 22/11/46 | 3 FW ewes | .0030 | + | +++ | − | | | | − | |
| Dispersible Benzene hexachloride and Derris | 22/11/46 | 3 FW ewes | .0015 | + | ++ | − | − | − | +(1) | | |
| Dispersible Benzene hexachloride Powder | 22/11/46 | 3 FW ewes | .0125 | + | +++ | − | | − | − | | |
| | ,, | 3 FW ewes | .0030 | + | ++ | − | | | | | |
| | 4/2/47 | 104 FW ewes 102 lambs | .0030 | +++ | − | − | | | | | |
| | 26/2/47 | 3 FW wethers | .0030 | + | ++ | − | − | − | − | | |
| | 16/6/47 | 2 CW ewes | .0012 | + | + | − | | | | | |
| | ,, | 2 CW ewes | .00060 | ++ | +++ | − | | | | | |
| | ,, | 2 CW ewes | .00030 | + | ++ | + | | | | | |
| | 10/7/47 | 2 CW hoggets | .00030 | ++ | − | + | | | | | |

## DISCUSSION

With regard to derris, the present work together with field data over several years has served to confirm the extreme toxicity of either derris powder or of rotenone to keds. Not only do they destroy all living keds at dipping but they remain in the fleece for a sufficient time to kill all young keds emerging from pupae. Provided the flock is not subjected to reinfestation with keds from undipped sheep, one dipping suffices to eradicate keds entirely. It follows that many of the materials at present incorporated in compounded dips are superfluous. A further point in favour of derris is that it is non-poisonous and is much cheaper than any other known dip.

On the other hand it must be admitted that derris does not give the same control over lice. In all cases derris kills all adult lice. The results show that the sheep remain apparently free of lice for several months and that in some cases they then suddenly reappear. Why they should not appear, often until 9-10 months after dipping, is not known. Since the examination of the sheep is very thorough, it can scarcely be due to the louse eggs hatching within a few weeks of dipping or the young lice would have been observed earlier. No explanation of this can be given. With most lice such as human lice and pig lice, the period of incubation is only a few weeks. In this respect very little is known of the life history of the sheep louse, so it is difficult to discuss what factors cause the reappearance of lice after such long periods. What is known, from experiments at the College, is that keds and particularly lice on isolated sheep vary in number from time to time with the nutrition and thrift of the host, and with seasonal conditions. In one case, two lice-infested sheep in low condition were found to be free of lice after shearing and an improvement in condition was noted. This, however cannot alter the fact that derris does not completely control lice.

The other disadvantage of derris, namely that it sometimes causes lameness, is also unexplained. The lameness is of a temporary nature appearing within a few days after dipping and rarely lasts for more than 2-3 weeks (2). No mortality occurs but it gives the sheep a setback. Outbreaks occur sporadically, affect some flocks and not others, and affect varying numbers within a flock. The only clue known at present is that it seldom if ever occurs in sheep dipped on the same day as the dip is made up, but occurs more frequently on subsequent days, or after large numbers have been put through the dip. As far as is known, derris extracts containing rotenone do not cause lameness.

The future of derris powder as a dip remains uncertain so long as the lameness problem is unsolved. Even with lameness and incomplete control of lice, derris still has a place until the ideal dip is discovered. For instance, the number of flocks infested with lice is small, and derris is so cheap and effective against keds that its use will probably continue. Where it is easy to empty and refill the dip in order to use a fresh derris dip each day, derris is safe and reliable. For dipping ewes with lambs it is certainly safer than the standard arsenic and phenolic dips.

The results with bentonite-sulphur were disappointing in view of the claims made for it. This can hardly be due to the method of preparation, and the fact that recently imported material contains rotenone leads one to suspect that the American workers have subsequently been unable to substantiate their original claims.

D.D.T. gave effective control of keds, thus confirming the observations of Heath (4). The concentration of D.D.T. might possibly be

reduced below Heath's lowest concentration of 0.125 per cent. since our results show control of keds down to a D.D.T. concentration 0.02 per cent. Some greater margin of safety may, however, be necessary, for at 0.01 per cent. ked control was not achieved. Unfortunately its efficiency in lice control is not good, in fact it is less effective than derris. Though a 100 per cent. initial kill was invariably achieved, lice reappeared within 2-3 months. Therefore like derris, it cannot be considered as a general purpose dip for both keds and lice but could be used for flocks infested with keds only. As far as is known D.D.T. is harmless to sheep but insufficient field data is available. Only large scale practical use can prove whether or not it will exhibit any harmful effects. If it does not there may be a place for it as a non-toxic, safe dip for ked infested flocks.

Benzene hexachloride has given very promising results. Complete control of keds was achieved at all concentrations down to 0.0006 per cent. It would be advisable to use a considerably stronger dip than this however, to give a margin of safety, to take account of the stripping out of the benzene hexachloride as the sheep are dipped, and to confer on the sheep some period of freedom from reinfestation. Until more experience is gained a concentration of the order of 0.003 can safely be recommended and will ensure complete control at one dipping together with 3-4 months freedom from reinfestation. A dip at such strengths should be capable of manufacture at a relatively low cost.

The effect of benzene hexachloride on lice is certainly better than that of any of the other materials tested. The initial kill was in all cases 100 per cent. With one exception no lice have reappeared though some of the sheep have been dipped for up to 12 months. The sole exception was in one of three ewes dipped in a derris—benzene-hexachloride mixture at a concentration of 0.0015 per cent. where live lice have been found. It appears therefore that benzene hexachloride is also very effective against lice. This, nevertheless, must be accepted with some caution yet, for experience with derris has shown that there is still time for lice to reappear. In addition, large scale trials against lice have not been possible owing to the difficulty of locating lousy flocks. If the effectiveness of control of lice is confirmed, benzene hexachloride promises to provide a single component dip which will control both keds and lice. Our experiments do not differentiate between emulsion or suspension forms and it is probable that both have roughly equal potentialities.

During last autumn several flocks have been dipped in a commercial dip containing benzene hexachloride and phenol. It was with some astonishment that reports were received of lameness with this dip. McLean (2) has investigated these and has found that the condition is apparently identical with the derris lameness but the outbreaks are less frequent and not so widely distributed. Thus, whilst benzene hexachloride shows every promise of being an outstanding material as a sheep dip there is still this problem to be overcome before its use can be advocated with complete confidence.

There is one further point about both D.D.T. and benzene hexachloride which is brought out by Australian workers (private communication N.P.H., Graham) but which has not been studied here. That is the 'stripping out' effect, whereby the D.D.T. or benzene hexachloride is taken out of the dip by absorption on the wool as the sheep

are dipped. This results in the concentration in the dip falling as more sheep are put through. The optimum particle size in the dip, the rate of topping-up with more D.D.T. or benzene hexachloride have still to be decided.

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## ADDENDUM

After twelve months had elapsed since the benzene hexachloride trials at strengths of 0·003 per cent. and greater (Table IV) against lice, no lice had appeared confirming that at these strengths the dip is effective. At the very weak concentrations there were still no lice after 8 months at dipping strength of 0·0012 per cent., but lice had appeared and multiplied after 4 months on those dipped at 0·0006 per cent. A flock of 350 heavily louse-infested sheep had been dipped under practical conditions at a strength of 0·003 per cent. and no lice had reappeared after 6 months.

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